THE "PINK SPOT" - URINARY EXCRETION OF VARIOUS SUBSTANCES BY SCHIZOPHRENICS AND CONTROL SUBJECTS

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The thesis is divided into two sections.

Section A deals with the pink spot phenomenon. Sub-Section I describes the development of a suitable urine extraction procedure, Sub-Section II details the application of this method to a screening survey of patients, Sub-Section III reports the refinement of the technique and the development of a parallel extraction method and in Sub-Section IV is discussed the final application of the procedures to a more detailed study of a small number of patients.

Section B deals with the urinary excretion of p-tyramine, tryptamine, 5-HT, IAA and 5-HIAA by the subjects of the final study.

Dr. J. R. Smythies and Dr. R. J. Daly assessed the patients clinically.

I undertook all of the laboratory work reported in Section A. Of that referred to in Section B I standardised the details of the procedures to be adopted in our laboratory for the preparation of samples and extraction and estimation of p-tyramine, tryptamine and 5-HT. Thereafter I prepared all samples, hydrolysed and unhydrolysed, and Mr. Ian Smith routinely extracted the amines by ion-exchange and estimated them fluorimetrically. All procedures concerning the acids were carried out by me.

Dr. G. W. Ashcroft gave advice on how the laboratory procedures might be conducted. Once I became acquainted with the basic techniques, I was able to plan many of the investigations undertaken.

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SECTION B

URINARY EXCRETION OF SOME AMINES AND ACIDS OF PHYSIOLOGICAL IMPORTANCE BY SCHIZOPHRENIC AND HEALTHY SUBJECTS

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SUMMARY

It has been proposed by some investigators that excretion of pink spot material is specific for schizophrenia and may have aetiological significance.

Two methods devised by other workers for the extraction of pink spot substances from the urine were examined. One was selected and somewhat altered. Employing this procedure forty psychiatric patients, half of whom were schizophrenic, were investigated. Pink spot material was detected in the urine of some of these subjects, but the incidence favoured neither group. The method was further altered and an additional means of urine extraction, already used by other investigators, was studied and modified. Employing both methods of urine extraction in parallel a smaller number of patients and also healthy control subjects were investigated. It was found that, contrary to what had previously been supposed, the pink spot material obtained by each method was different. The substance obtained by a solvent extraction procedure was not 3,4-dimethoxy phenylethylamine, p-tyramine or tryptamine as proposed by other workers, but had properties in common with monoacetyl cadaverine. The detection of this material in the urine of all apparent non-excretors, schizophrenic or otherwise, could be effected by increasing the fluid intake and consequent urinary output of the subjects. Two compounds were detected in the urine of all subjects when an ion-exchange extraction method was employed. One of these was p-tyramine but the other was not identified.

The urinary excretion of p-tyramine, tryptamine, 5-hydroxytryptamine, indole-acetic acid and 5-hydroxyindoleacetic acid by the smaller number of subjects was also examined. The finding that schizophrenic patients

excrete more p-tyramine than other subjects was not confirmed. The established dependence of excretion of indole-acetic acid on urinary pH was confirmed and this was also found to be the case for tryptamine, a fact not previously recorded. Since an increased excretion of tryptamine and indole-acetic acid by schizophrenic subjects has been noted by various investigators it may be of significance that the pH dependence of these substances was not taken into account in their studies.

GENERAL INTRODUCTION

There appears to be general agreement that schizophrenia is a syndrome rather than a disease entity. However this is often lost sight of by clinical investigators and the inclusion of patients with such dissimilar conditions as paranoid schizophrenia, catatonic schizophrenia and simple schizophrenia in supposedly homogeneous samples undoubtedly accounts in part for the lack of significant advance in knowledge of the aetiology of those illnesses since the term "The Schizophrenias" was first used by Bleuler in 1911.

Some psychiatrists consider that psychological stress is wholly responsible for the occurrence of this group of diseases while others attach little or no importance to such factors. Perhaps the majority of clinicians favour a synthesis of theories ascribing aetiological significance to physical and psychological influences. A currently acceptable hypothesis is that there is a genetic predisposition to those conditions, that their onset is at times preceded by psychological stress and that although the form of the symptoms is not in any way related to such stress the content of such mental aberrations does derive from the individual patient's experiences.

Although genetic abnormalities seem likely the finer biochemical mechanisms involved remain unknown despite a considerable amount of investigation over many years. The difficulties inherent in such research are many and it is probable that the pitfalls awaiting the unwary are more legion in the study of chronic mental illness than in the conduct of enquiry into non-psychiatric disease. Some of the sources of error

have been outlined by Kety (1959). Most schizophrenic patients studied have lived institutionalised lives for many years and this mode of existence brings in its train several factors likely to interfere with the researcher's enquiries. Chronic infections, in particular of the gastro-intestinal tract, find their origin in overcrowding and inadequately maintained hygiene. This induced development of a characteristic intestinal flora may lead to apparently deranged metabolic pathways i.e. the atypical metabolites are produced by the bacteria rather than by the host and do not affect the health of the latter. Seeming biochemical abnormalities have finally been attributed to dietary deficiency or idiosyncrasy on more than one occasion. Acute and chronic emotional stress, inherent in mental illness, have their physical concomitants which, unless recognised as such, are liable to be interpreted in terms of primary rather than secondary deviation from physiological normality. The inevitable resort to the use of drugs for the control of schizophrenic symptoms may lead to similar misinterpretation. By no means the least important factor responsible for erroneous conclusions is subjective bias. That this should be present is not surprising when one considers that the condition is so variable and so responsive to non-specific factors in the environment and that, as Kety points out "motivation (is) heightened by the tragedy of this problem and the social implications of findings which may contribute to its solution".

If the study to be described has succeeded in avoiding some of the possible errors of methodology and experimental design it is because the investigator has had the advantage of noting those committed by previous workers in this field and not because of any particular excellence in the execution of research methods.

SECTION A

INTRODUCTION

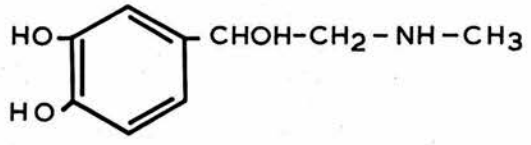
3,4-dimethoxyphenylethylamine and the "pink spot" phenomenon

That knowledge gleaned by study of the psychoses produced by toxic substances may illuminate the obscure mechanisms concerned in schizophrenic disorders has been recognised for over a hundred years. Moreau de Tour (1845) published a monograph entitled "Du Hachich et de L'Alienation Mentale. Études psychologiques" and mescaline was considered in this light by Prentiss and Morgan (1895) and by Beringer (1927). Once the chemical structure of mescaline had been established by Spaeth (1919) the way was open for speculation as to the possible significance of the chemical similarity of mescaline and adrenaline (Fig. 1).

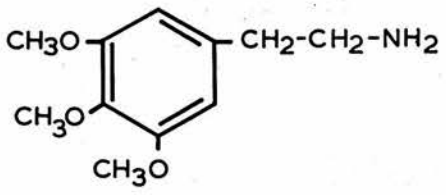
De Jong (1931) demonstrated that both mescaline and adrenaline were capable of producing "experimental catatonia" in different animal species and Noteboom (1934) investigated the catatonic properties of a series of substances chemically allied to mescaline, among them 3,4-dimethoxyphenylethylamine (Fig. 1). Almost twenty years later Mayer-Gross (1951) in discussing experimental psychoses again drew attention to the close chemical relationship between mescaline and adrenaline and in 1952 on the basis of this structural resemblance, a hypothesis was advanced by Osmond and Smythies (1952). It was proposed that schizophrenia might be a disorder caused by abnormal metabolites of adrenaline, these substances being even more akin to mescaline than adrenaline itself and having the same psychotogenic properties as the hallucinogen. In the same paper the chemist Harley-Mason suggested that the phenolic hydroxyl groups of adrenaline might be methylated and 3,4-dimethoxyphenylethylamine was specified as

Fig. 1

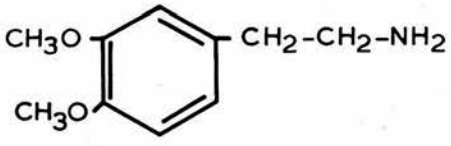
Chemical structure of adrenaline, mescaline and d.m.p.e.



Adrenaline



Mescaline



3,4 – dimethoxyphenylethylamine

a likely product.

Osmond and Smythies had formulated their thesis at a time when nothing was known about the methylation of adrenaline so that the discovery by Axelrod, Senoh and Witkop (1959) that the first step in the conversion of adrenaline and noradrenaline to their acid metabolite was the substitution of a methoxy group for one of the ring hydroxyl groups was of considerable interest. However in the following few years it was shown that schizophrenic and normal subjects excreted similar quantities of adrenaline, noradrenaline (Bergsman 1959) and of vanilmandelic acid, the main metabolite of the two amines (Mann and La Brosse 1959; Pind and Faurbye 1961) and that when tritium-labelled adrenaline was administered intravenously to schizophrenic and normal persons the same proportions of metabolites were detected in blood and urine irrespective of clinical state (La Brosse, Mann and Kety 1963). Nevertheless the formation of small amounts of an abnormal metabolite in the tissues of schizophrenic subjects could not be excluded.

In 1962 Friedhoff and Van Winkle (1962) announced that they had detected, in the urine of schizophrenics, but not of controls, a substance which they had identified as 3,4-dimethoxyphenylethylamine (d.m.p.e.). Employing one-way paper chromatography of a chloroform extract of urine a chromatographic pink spot, isographic with d.m.p.e. in several solvent systems, was obtained following treatment of the paper sequentially with ninhydrin and p-dimethylaminobenzaldehyde. This material and d.m.p.e. also appeared to form identical derivatives with two groups of reagents. In 1963 investigations by these same workers indicated that tritiated dopamine administered to schizophrenics was converted to d.m.p.e. and its

acid derivative and that biopsied liver tissue from such a patient could convert dopamine to d.m.p.e. in vitro (Friedhoff and Van Winkle 1963a). In further support of the identification of this substance sufficient was isolated to allow of the determination of its melting point, identical with that of d.m.p.e. (Friedhoff and Van Winkle 1964). At a symposium (Friedhoff and Van Winkle 1965) they reported that they had characterised this substance by means of thin layer chromatography and gas liquid chromatography and Friedhoff and Furiya (1967) confirmed the original finding of d.m.p.e. only in the urine of schizophrenics.

This apparent substantiation of the Osmond, Smythies, Harley-Mason hypothesis was naturally followed by intensive activity on the part of other scientists. Many criticised the use of the one-way paper chromatographic system because clear isolation of the several substances encountered was not satisfactory and therefore various techniques of urine extraction were evolved.

That the 'pink spot' substance occurs only in the urine of schizophrenic patients has been disputed by Takesada et al (1963) Von Studnitz and Nyman (1965) and Vogel et al (1967) although Bourdillon et al (1965) and Predescu et al (1968) are in agreement with the original investigators. While Friedhoff and Van Winkle studied subjects with acute schizophrenic symptoms these other authors among them reported 'pink spot' precursors to be excreted by patients suffering from both the acute and chronic forms of the disease.

There has been disagreement not only as to the specificity of excretion of the 'pink spot' substance for schizophrenia but also as to its chemical nature. Claim for its identity as d.m.p.e. has been

supported by Takesada et al (1963) Sen and McGeer (1964) Kuehl et al (1964) Von Studnitz and Nyman (1965) and Vogel et al (1967) while Bourdillon et al (1965) and Predescu et al (1968) do not definitely claim that their chromatographic pink spots were derived from d.m.p.e. Bell and Somerville (1966a) and Boulton and Felton (1966) excluded d.m.p.e. as a constituent of the pink spot precursors they observed and Faurbye and Pind (1964 and 1966), Perry et al (1964) and Williams et al (1966) state that they were unable to find evidence for the presence of this amine in the urine of the schizophrenic subjects studied. Bell and Somerville (1966b) have developed a much more specific method for the identification of d.m.p.e. than most others available but unfortunately only three groups have reported the application of this procedure. In two instances the results were negative (Bell and Somerville 1966a, Boulton and Felton 1966) but Rinne and Sonninen (1967) found small amounts, insufficient to yield pink spots, in the urine of patients with Parkinsonism and various other neurological diseases. Employing the most definitive means of exploring chemical structure but unfortunately one which requires more material than most investigators have isolated, Creveling and Daly (1967) obtained a mass spectrogram of d.m.p.e. from purified urine extract. This material was present in the d.m.p.e. marker zone of a chromatogram of urinary extract from schizophrenic subjects but the quantity was not sufficient to give rise to a pink spot with the reagents cited by Friedhoff and Van Winkle. While not denying the presence of pink spot producing material in the urine of schizophrenic and other subjects Perry et al (1967) considered the compounds they observed to be monoacetyl and monopropionyl cadaverine and Boulton et al (1967) reported p-tyramine to be responsible for the

pink zone obtained. Sen and McGeer (1964) found not only d.m.p.e. in the urine they extracted but also 4-methoxyphenylethylamine, another methylated phenylethylamine giving the 'pink spot' reaction. One group recently proposed that tryptamine contributes to the colour of the pink spot (Kuehl et al 1968).

Pink spot reacting compounds have been considered to be endogenous (Friedhoff and Van Winkle 1964), dietary (Perry et al 1964, Von Studnitz and Nyman 1965) or bacterial in origin (Perry et al 1966) or to be the result of drug administration. Steinberg and Robinson (1968) suggested that d.m.p.e. was present in the material yielding the pink spot detected by Friedhoff and Van Winkle but that the major component was nor_2chlorpromazine sulphoxide. Takesada et al (1963) identified this drug metabolite on a urinary paper chromatogram and noted that it could be confused with d.m.p.e. as it also gives the pink spot reaction and migrates in a similar fashion in several chromatographic solvent systems. Clos et al (1967) also found pink spots on paper chromatograms to be correlated with psychotropic drug administration. Most investigators have withheld drugs during their investigations but perhaps not for sufficiently long periods beforehand since chlorpromazine metabolites continue to be excreted for several months (Jarvik 1965).

The investigators cited have not employed identical methods for extracting urine. Various solvent extraction techniques were adopted by Friedhoff and Van Winkle (1962), Sen and McGeer (1964), Bourdillon et al (1965), Bell and Somerville (1966a) and Creveling and Daly (1967) while adsorption on cation exchangers was favoured by Takesada et al (1963), Faurbye and Pind (1964), Kuehl et al (1964), Perry et al (1967) and

Boulton et al (1967).

The majority of experimenters have directed their attention to identification of urinary constituents but Friedhoff and Van Winkle's other claims have not gone entirely unchallenged. Thus Faurbye and Pind (1967) failed to detect radioactive d.m.p.e. in the urine of schizophrenic and normal individuals to whom tritium labelled dopamine had been administered and Kuehl et al (1966) could not demonstrate the conversion of dopamine to d.m.p.e. when the former was incubated with rat liver. This latter situation is not, of course, strictly analogous to the experiment with biopsied human liver which Friedhoff and Van Winkle performed (1963a).

Strangely it was not until 1966 that d.m.p.e. was taken by human subjects. Neither Hollister and Friedhoff (1966) nor Shulgin et al (1966) noted psychotic or other symptoms in normal individuals or patients who had previously suffered a "schizophrenic reaction".

PURPOSE OF THE STUDY

The purpose of the study was to determine whether or not schizophrenic and other patients in this hospital community excreted 'pink spot' precursors and, if they did, to examine the identity and possible significance of those substances. Our own preliminary studies and the reports of others pointed to the desirability of comparing the 'pink spot' reacting materials obtained by more than one urine extraction technique.

SUB-SECTION 1

DEVELOPMENT OF A SUITABLE URINE EXTRACTION PROCEDURE

1. Comparison of the Solvent methods of Friedhoff and Van Winkle and Bell and Somerville.

a) Partition coefficient of d.m.p.e.

The essential difference between the two methods was the use of different organic solvents, chloroform by Friedhoff and Van Winkle and dichloroethane by Bell and Somerville. In order to determine whether the use of dichloroethane conferred any advantage the partition coefficient of d.m.p.e. between buffer solutions and these solvents was estimated, the effect of saturation of the aqueous phase with sodium chloride also being examined.

0.2 M glycine-sodium hydroxide buffers having different pH values were prepared. The pH values chosen were 8.6, 9.0, 9.6, 10.0 and 10.6 in order to compare the results obtained by Bell and Somerville. D.m.p.e. was added to each to achieve a final concentration of 100µg/ml. Four 9ml. aliquots of each solution were taken and the individual portions within each set of four were to be treated as follows :- a) extracted with 3ml. chloroform, b) salt-saturated and extracted with 3ml. chloroform, c) extracted with 3ml. dichloroethane and d) salt-saturated and extracted with 3ml. dichloroethane.

Blank (i.e. not containing d.m.p.e.) samples of buffer pH 9.6, salt-saturated buffer pH 9.6, chloroform and dichloroethane were also prepared as was a standard solution of d.m.p.e. 100µg/ml. in water.

The buffer samples, other than the blank, were shaken for four minutes with the appropriate solvent, centrifuged and the phases separated. The ultra-violet absorbance of organic and aqueous samples were estimated

in an S.P800 spectrophotometer. The d.m.p.e. peak was 277mμ as compared with 279mμ quoted by Bell and Somerville (1966a).

Results

The values given in Table 1 are the ultra-violet absorbances at 277mμ of each sample minus that of the appropriate blank.

It will be seen from Table 2 that the partition coefficient $\frac{\text{concentration of d.m.p.e. in organic solvent}}{\text{concentration of d.m.p.e. in buffer}}$ increased with pH, that salt-saturation improved extraction of d.m.p.e. into both solvents and that chloroform was a better organic solvent of d.m.p.e. than dichloroethane.

These findings influenced the final choice of the method of Friedhoff and Van Winkle for the screening survey. However, it was not felt that salt saturation offered sufficient advantage to offset the inconvenience involved in this procedure.

b) Estimation of the Recovery of d.m.p.e. from urine employing the unmodified methods

i. Friedhoff and Van Winkle's procedure

To 300ml. urine was added a solution of barium chloride until no further precipitation occurred. After filtering, the urine was adjusted to pH 2.0 with hydrochloric acid and extracted three times with 150ml. chloroform to remove acids, the chloroform discarded, the urine filtered again, adjusted to pH 9.0 with 2N sodium hydroxide and extracted a further three times with 150ml. chloroform. These combined chloroform extracts were dried by the addition of anhydrous sodium sulphate and reduced to a suitable volume for paper chromatography.

TABLE 1

DATA FOR CALCULATION OF PARTITION COEFFICIENT OF D.M.P.E.

ULTRA-VIOLET ABSORBANCE

pH	Dichloroethane		Dichloroethane +Salt-Saturation		Chloroform		Chloroform +Salt-Saturation	
	Organic	Aqueous	Organic	Aqueous	Organic	Aqueous	Organic	Aqueous
8.6	0.54	0.82	1.04	0.78	3.00	0.32	2.92	0.22
9.0	1.42	0.80	1.74	0.72	3.34	0.20	4.54	0.13
9.6	2.00	0.43	2.38	0.24	3.10	0.10	2.92	0.04
10.0	2.48	0.40	2.87	0.13	3.58	0.10	3.58	0.02
10.6	3.12	0.30	3.60	0.10	3.76	0.08	4.00	0

TABLE 2

PARTITION COEFFICIENT OF D.M.P.E. ORGANIC/AQUEOUS

pH	Dichloroethane buffer	Dichloroethane / Salt Saturated buffer	Chloroform buffer	Chloroform / Salt Saturated buffer
8.6	0.66	1.33	9.4	13.3
9.0	1.77	2.40	16.7	35.0
9.6	4.65	9.9	31	73
10.0	6.20	22	36	179
10.6	10.4	36	46	∞

The coefficients have been derived from the data in Table 1.
No allowance has been made for differences, if any, between the curves relating absorbance to concentration in organic and aqueous phases.

The organic phase was ⅓ vol. of aqueous phase as in Friedhoff and Van Winkle's procedure but this inequality was not taken into account in calculating the coefficients.

ii. Bell and Somerville's Extraction Procedure

300ml. urine was extracted three times at pH 10.0 with volumes of dichloroethane equal to one-third of that of the urine. Residual aqueous phase was removed from the solvent by the addition of anhydrous sodium sulphate and the extract reduced to dryness by vacuum distillation in a rotary evaporator following which the residue was dissolved in 0.01 N hydrochloric acid and applied to Whatman No.1 chromatography paper.

iii. Chromatography

Bell and Somerville did not alter Friedhoff and Van Winkle's chromatography procedure. This entailed the use of Whatman No.1 paper for ascending chromatography in n-butanol-acetic acid-water (4:1:1 by volume) for 16 hours.

iv. Estimation of d.m.p.e. by the 'Pink Spot' Reaction

Friedhoff and Van Winkle employed a colour reaction for the detection and quantitative estimation of d.m.p.e.

Following chromatography the dried paper was dipped through 0.2% ninhydrin in acetone-pyridine (9:1 by volume), air-dried, heated in an oven at 105°C for 1 min., dipped in 2% p-dimethylaminobenzaldehyde in acetone-conc. hydrochloric acid (9:1 by volume) and examined for pink and other colours after a further 30 mins.

Friedhoff and Van Winkle (1963b) submitted a number of compounds to this procedure and concluded that the pink colour was produced only with β-phenylethylamine, β-phenylethylamines substituted on the ring and β-phenylethylamino-acids. Substitution on the side chain decreased the sensitivity of the test. They did not attempt to elucidate the mechanism

of the reactions involved but suggested that Ruhemann's Purple formed by interaction of ninhydrin and the aromatic amine reacted further with p-dimethylaminobenzaldehyde to give the pink-coloured substance.

After drying the paper Friedhoff and Van Winkle eluted the pink-coloured material with 75% acetone-water and measured the absorbance at 575mμ.

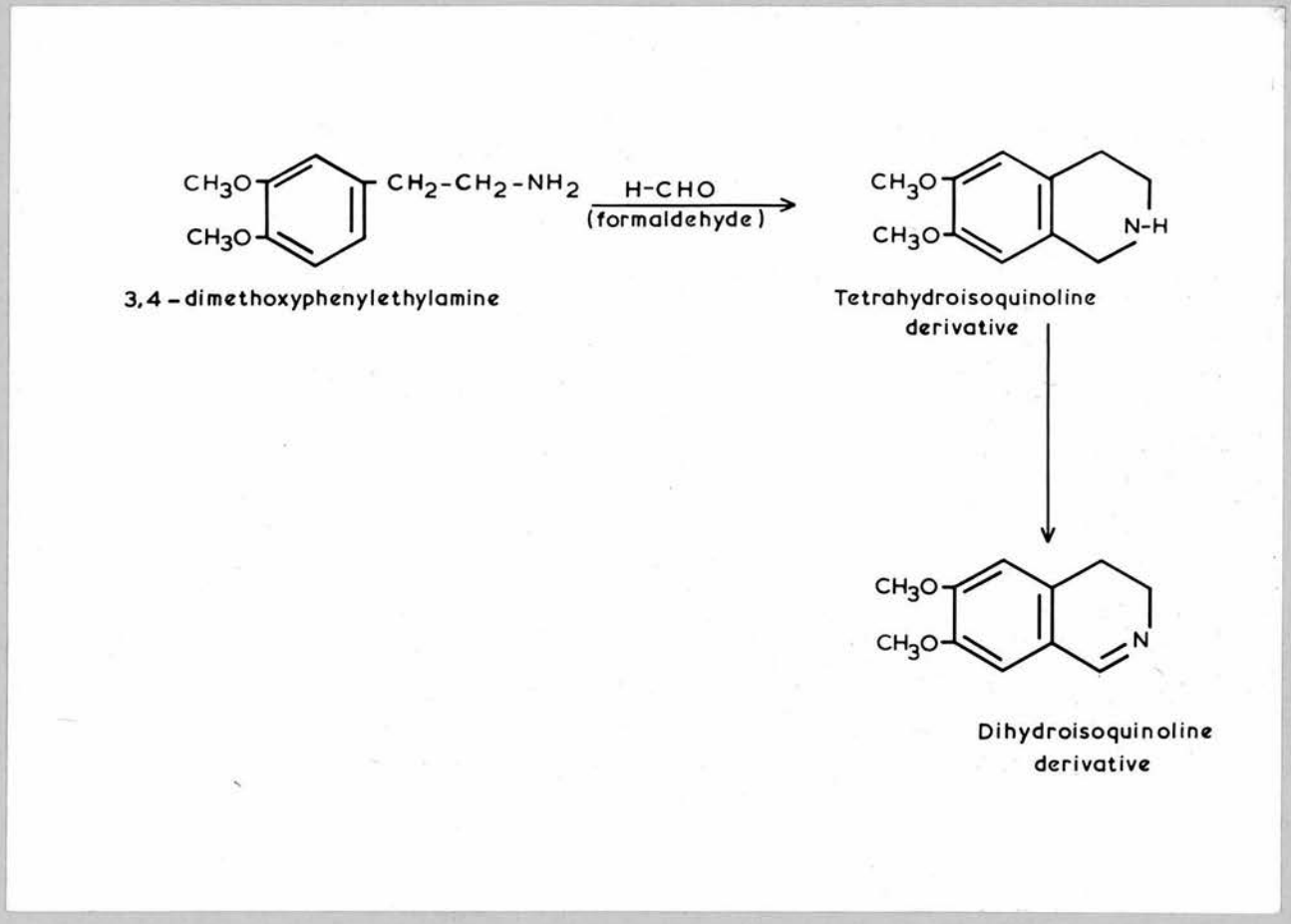
The final quantitative step was omitted in this study as the pink colour was not specific for d.m.p.e. and other positively reacting substances would augment the true d.m.p.e. value. Moreover, it was considered that the fluorescence method of Bell and Somerville would be more sensitive and more accurate.

v. Measurement of d.m.p.e. by Fluorescence

Quantitative measurement of d.m.p.e. was by the procedure developed by Bell and Somerville (1966b). This depended upon the condensation of a substituted phenylethylamine (in this case d.m.p.e.) in the presence of formaldehyde to form a tetra-hydroisoquinoline and subsequent dehydrogenation to the fluorescent dihydroisoquinoline (Fig. 2). Following chromatography, the paper was sprayed with 5% glycine adjusted to pH 3.0 with hydrochloric acid, oven dried at 80°C before being suspended in a sealed Kilner jar containing 20g. paraformaldehyde moistened with 6ml. water, heated in an oven at 80°C for 3 hrs. and viewed under an ultra-violet source. The fluorophor thus located was eluted from the paper overnight with 3ml. 0.4M sodium hydroxide in methanol. The eluate was acidified with 0.5ml. conc. hydrochloric acid and the spectral characteristics of the fluorophor determined in a spectrophotofluorimeter. Either an Aminco-Bowman or a Farrand instrument was used.

Fig. 2

Steps in the formation of the fluorescent product of d.m.p.e.



Results

Location of d.m.p.e. by the 'pink spot' reaction

Chromatograms were of poor quality in that there was inadequate separation of ninhydrin reacting compounds and considerable retardation of d.m.p.e. added to the urine compared with marker d.m.p.e. in pure solution. This appeared to be due to extraction of pigment from urine. This pigment was observed as a brown colour on the paper before ninhydrin treatment.

Recovery of d.m.p.e. estimated by the Fluorescence Method

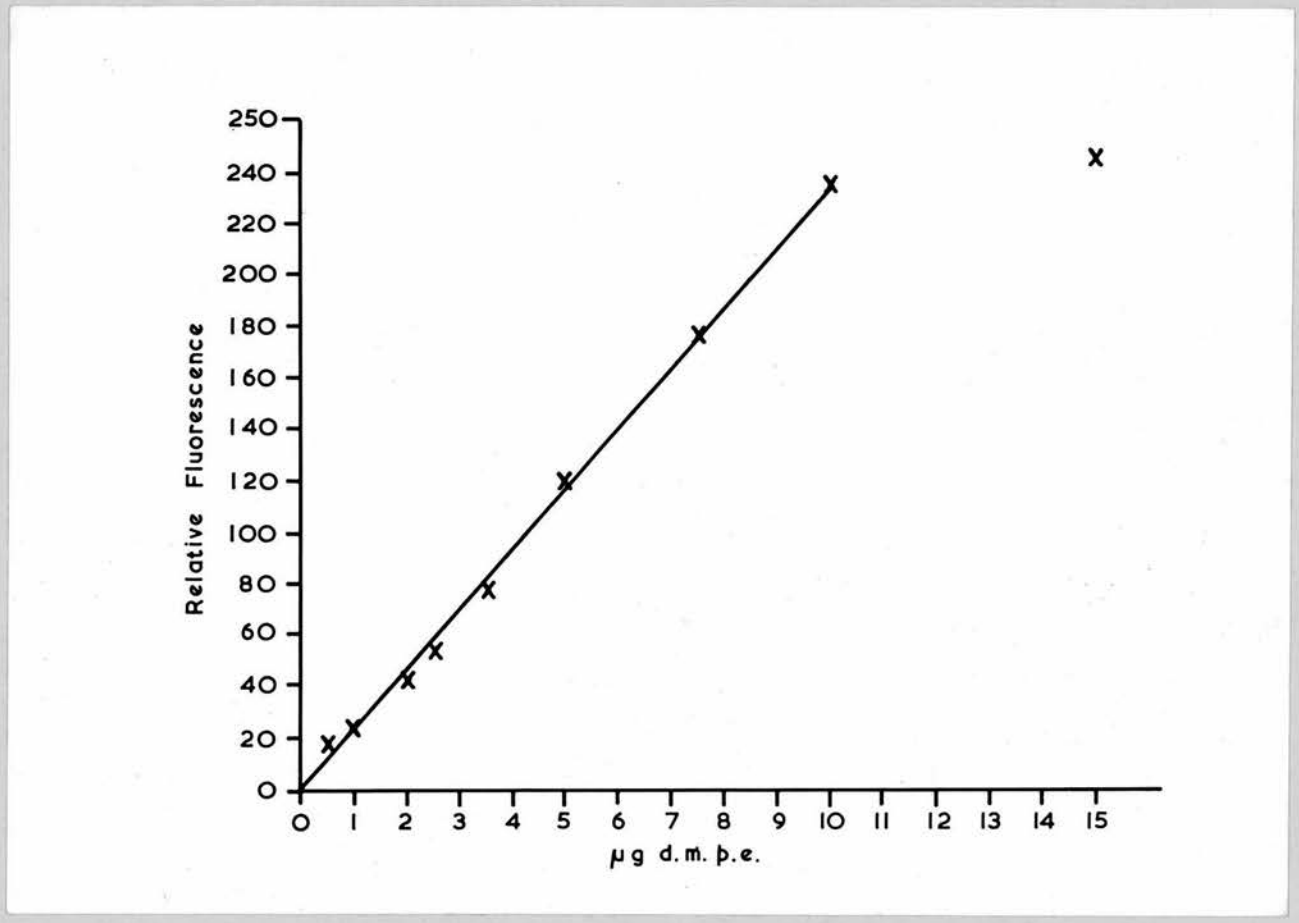
D.m.p.e. gave an ice-blue fluorescence when viewed under ultra-violet light and fluoresced maximally at 480mμ when excited at 365mμ during this study. Excitation and fluorescence maxima quoted by Bell and Somerville are 360 and 470mμ respectively. Other substituted phenylethylamines and indolealkylamines yield different fluorophors, with the exception of 3-hydroxy-4-methoxyphenylethylamine. The fluorescent substance derived from this compound gave a similar colour and although Bell and Somerville quoted a fluorescence maximum at 465mμ compared with 470mμ for d.m.p.e. it would not have been possible to distinguish between them.

In pure solution as little as 0.03μg d.m.p.e. yielded detectable fluorescence after the reaction. Linearity was tested from 0.5 - 15μg. The fluorescence was linear from 0.5 - 10μg. (Fig. 3).

Although Bell and Somerville employed paper chromatography they finally applied their method (1966b) to the estimation of d.m.p.e. which had been separated from other extracted urinary constituents by high voltage electrophoresis (1966a). In the study reported here, several difficulties were encountered when attempting to calculate recovery of

Fig. 3

Calibration curve for d.m.p.e. estimated by the method of Bell and Somerville



d.m.p.e. added to urine. The presence of other substances at the same location as the d.m.p.e. fluorophor partially masked the fluorescence, streaking of the fluorescent material was caused by the presence of pigment and widely varying blank values (fluorescence at the optimal wavelengths for d.m.p.e. obtained from areas of paper not containing d.m.p.e.) diminished the accuracy of the results.

The quantity of d.m.p.e. present in the chromatographed urine extract was calculated by subtraction of the amount of fluorescence produced by a section of the chromatogram which did not contain d.m.p.e. from the d.m.p.e. fluorescence and comparison of this value with a calibration curve constructed from values obtained for different amounts of d.m.p.e. applied to the paper in pure solution and submitted to the reaction in parallel.

Several experiments employing either extraction procedure indicated that of 30µg d.m.p.e. added to 300ml. urine, only about 10µg were retrievable. This was considerably below the 50-60% recovery which Bell and Somerville reported in their paper (1966a). However, it was in keeping with values obtained separately by them and by me during a three week period which I spent in their laboratory.

The similarity of the efficiency of each method seemed to indicate that the advantage conferred by the superior solubility of d.m.p.e. in chloroform, compared with that in dichloroethane, was offset by losses during other stages of the Friedhoff and Van Winkle procedure.

2. Modification of the Procedure of Friedhoff and Van Winkle

As the recovery of d.m.p.e. employing either of the two methods examined was unsatisfactory, an attempt was made to improve the

Standard extraction procedure of Friedhoff and Van Winkle

300ml urine + solution of barium chloride until precipitation ceases

↓

Filter urine

↓

Adjust to pH 2.0 with conc. hydrochloric acid

↓

Extract 3 times with 150ml chloroform

↓ → discard chloroform

Filter urine

↓

Adjust to pH 9.0 with 2 N sodium hydroxide

↓

Extract 3 times with 150ml chloroform

↓

combine chloroform extracts

↓

shake with sodium sulphate

↓

evaporate to about 0.1 - 0.2ml

↓

apply to chromatography paper

↓

Ascending chromatography overnight (about 16 hrs.) in n-butanol-acetic acid-water (4:1:1)

↓

dry paper

↓

dip through Ehrlich reagent → dry →

dip through ninhydrin reagent

efficiency of one of the procedures by the investigation of possible sources of loss at each stage of the process. The technique of Friedhoff and Van Winkle was chosen because of the more favourable partition coefficient of d.m.p.e. between chloroform and aqueous phase, because extraction of the urine at pH2 with chloroform removed acids which might otherwise have appeared on the chromatogram and because its authors had detected 'pink spot' material in the urine more frequently than had Bell and Somerville.

Methods

The alterations in detail were planned in the light of the knowledge that amines in dilute solution are unstable at elevated temperature, under conditions of high alkalinity and when exposed to oxidising influences.

To 300ml. urine was added a solution of saturated barium chloride until no further precipitation occurred. This step was probably dispensable but it was decided to modify the original procedure only if an improvement in recovery of amines could be expected.

a) Removal of Acidic Material Following filtration through Whatman No.1 paper and adjusting to pH2.0 (glass electrode) with conc. hydrochloric acid, the urine was shaken on three occasions for three mins. with fresh batches of 150ml. chloroform and the chloroform discarded after separation by centrifugation at 2,500 revs./min. for three mins.

b) Extraction of amines. Avoidance of prolonged exposure to alkaline conditions

After filtering to remove residual chloroform and emulsion, the urine was adjusted to pH10.0 with 2.5N sodium hydroxide. The use of a more concentrated solution was not considered advisable because of the resulting exposure of the dissolved amines to unnecessarily highly

Modified extraction procedure

300ml urine + solution of barium chloride until precipitation ceases

↓

Filter urine

↓

Adjust to pH 2.0 with conc. hydrochloric acid

↓

Extract 3 times with 150ml chloroform → discard chloroform

↓

Filter urine

↓

Adjust to pH 10.0 with 2.5 N sodium hydroxide

↓

Extract 3 times with 150ml chloroform, the first extraction immediately following pH adjustment

↓

Combine chloroform extracts

↓

Wash chloroform with 50ml 0.2M glycine-sodium hydroxide buffer pH 10.0 → discard buffer

↓

evaporate to 10ml at 32°C in an atmosphere of nitrogen

↓

add 0.2ml 20% acetic acid

↓

reduce volume to 0.05 - 0.1ml under jet of nitrogen

↓

dissolve in 0.06ml 80% methanol in water containing ascorbic acid

↓

apply to chromatography paper, drying under nitrogen

↓

flush chromatography tank with nitrogen

↓

Ascending chromatography overnight (about 16hr.) in n-butanol-acetic acid-water(4:1:1)

(saturated side-papers and sealed weighted lid on tank)

↓

dry paper with nitrogen

↓

dip through Ehrlich reagent → dry →

dip through ninhydrin reagent

alkaline conditions. Because of this susceptibility to chemical change at high pH values the first extraction with 150ml. chloroform was carried out immediately after pH adjustment. Extraction was for four mins. and the resulting emulsion required centrifugation at 2,500 revs./min. for 8-10 mins. The chloroform from three such consecutive extractions was combined.

c) Buffer Wash Since urinary pigment had caused unsatisfactory chromatographic separation in the preliminary experiments, a buffer wash was introduced at this stage. Only 50ml. was employed in order to avoid appreciable loss of d.m.p.e. That this aim was achieved was demonstrated by an experiment in which 100µg was added to urine and following the wash stage, the buffer was extracted three times with 25ml. chloroform, the pooled chloroform being reduced in volume and chromatographed. No d.m.p.e. was recovered as a pink spot from this chloroform extract. The buffer solution was 0.2M glycine-sodium hydroxide pH10.0. The effect of the wash depended upon the greater affinity of urinary pigment for water than for chloroform. Such washed extracts yielded more satisfactory chromatograms although concentrated urine samples still posed a problem.

d) Use of warm water bath. Minimisation of loss on glassware. The chloroform was dried by the addition of anhydrous sodium sulphate and reduced in volume by vacuum distillation. A rotary evaporator was employed, the rotating flask being warmed in a water-bath at a temperature not exceeding 32°C. In order to minimise adsorption of solutes to the interior of the flask samples were not reduced to dryness but to about 10ml. and the flask was rinsed with 5ml. chloroform which was added to the extract which had been transferred to a test tube.

e) Acid Conditions prior to Chromatography Lest any aqueous alkaline phase unavoidably carried over into the organic solvent produce a final solution of high pH owing to the earlier evaporation of the more volatile chloroform, 0.2ml. 20% acetic acid was added to the extract at this juncture, giving a final pH of 1-2 during the late stages of evaporation.

f) Nitrogen as an anti-oxidant Prior to vacuum distillation, the rotary evaporator was flushed out with nitrogen and following the reduction in volume the extract was further concentrated in a test tube by a stream of the inert gas.

g) Undesirability of evaporation to dryness As in the rotary flask the extract in the test tube was not evaporated to complete dryness since this would have exposed the amines to oxidation by atmospheric oxygen. Rather was the chloroform-acetic acid concentrated to a small drop which was then dissolved in 0.06ml. 80% methanol in water.

h) Ascorbic acid as an additional anti-oxidant Since amines were likely to be oxidised at any and every stage of the procedure, 0.05% (w/v) ascorbic acid was dissolved in the 80% methanol in water (ascorbic acid is insoluble in pure methanol) which was employed as indicated above. This final extract of amines dissolved in methanol-acetic acid was chromatographed.

i) Use of Nitrogen for Chromatography The extract was applied as a spot to Whatman No.1 chromatography paper. Point rather than strip application was preferred since the latter method would have spread the amines over a wider area and reduced the quantity which could be detected visually as a pink spot or a fluorophor. For the reasons already indicated, the extract being applied to the paper was dried under a jet of nitrogen.

The test-tube was washed with a further 0.03ml. methanol-ascorbic acid solution which in turn was applied to the paper.

Before allowing the chromatogram to develop overnight, the tank was flushed with nitrogen for 5-10 mins.

j) Saturation of the enclosed chromatography system with the vapour phase of the solvent

200ml. n-butanol-acetic acid - water (4:1:1 by volume) for each chromatography trough and a further 400ml., applied to the upper ends of sheets of Whatman No.1 paper suspended at each side of the tank, was used. In order to ensure maintenance of saturation of the atmosphere within the tank with the solvent vapour, the lid was weighted and a gas-tight joint between lid and tank achieved by applying glycerol.

k) Duration of chromatography and drying the paper The ascending chromatogram was developed for 16 hr. following which the paper was removed and dried in another tank with a rapid stream of nitrogen. This mode of drying was preferred to heating in an oven because of the instability of amines at elevated temperature.

Thereafter the paper was either developed for the 'pink spot' reaction, or d.m.p.e. fluorescence as already described. Since most investigators employed a temperature of 80°C rather than 105°C as specified by Friedhoff and Van Winkle for acceleration of the ninhydrin reaction, this former temperature was adopted and the duration of exposure increased to 1.5 min.

Results

Location of d.m.p.e. by the 'pink spot' reaction

The application of this method yielded better quality chromatograms in several respects. Firstly, pigment did not interfere to the same

extent. Secondly, many more ninhydrin reactors were observed. Presumably these substances had been destroyed in previous experiments due to lack of adequate precautions. Thirdly, added d.m.p.e. was not retarded to the same extent, although migration to a position behind d.m.p.e. applied in pure solution still occurred. Finally, whereas at times 15µg d.m.p.e. added to urine had not always shown up as a clearly defined spot when the unmodified methods were employed, 7.5µg could be detected as a pink spot when added to urine extracted by the improved procedure.

Recovery of d.m.p.e. estimated by the fluorescence method

The alterations in procedure were reflected in the improved recovery of d.m.p.e. added to the urine. There was less marked tailing of the fluorophor and although no satisfactory solution to the variable paper blank fluorescence was to hand this factor was somewhat less troublesome. Recovery of 7.5µg varied between 53% and 71%. This variable recovery and the knowledge that similar quantities of d.m.p.e. yielded different amounts of fluorophor on different occasions (Somerville personal communication) necessitated the construction of a calibration curve from values obtained from standards submitted to the reaction on each occasion.

Thus, although this method was more specific and sensitive than the 'pink spot' reaction for detection and measurement of d.m.p.e., its use in combination with paper chromatography was less satisfactory than when paired with high voltage electrophoresis, the procedure preferred by Bell and Somerville.

The minimal quantity detectable was greater employing paper chromatography, i.e. the sensitivity of the method was impaired.

SUB-SECTION II

A SCREENING STUDY OF PATIENTS. APPLICATION OF THE URINE EXTRACTION PROCEDURE

Purpose of the study

This part of the study was intended as a screening survey designed to detect as many patients excreting 'pink spot' substances as possible following which a more detailed study would be undertaken. To this end it was decided to extract one third of each 24 hr. urine sample and chromatograph the whole extract despite the risk of overloading the paper.

Methods

Selection of subjects

Since very few acute schizophrenics, either untreated or treated, were available chronic schizophrenics were chosen as subjects. Twenty schizophrenic and twenty non-schizophrenic psychiatric patients were selected, the sexes being equally represented. The age range of the schizophrenics and controls respectively was 26-69 years and 17-77 years and the mean age 52.8 years and 47.5 years. Range of duration of stay in hospital prior to the study for schizophrenics was 7 days to 34 years and the mean duration of stay 3.4 years. For control subjects, duration of stay was 2 days to 44 years and the mean duration of stay 7.9 years.

All schizophrenic patients were so judged on the basis of their current clinical status and previous history by an independent psychiatrist and by the psychiatrists who co-operated in this study. Although all had at one time active symptoms of schizophrenia, one out of the 10 women and 6 out of the 10 men manifested only withdrawn, apathetic behaviour with emotional blunting, at the time of screening. Of the 9 other women, 8 were hallucinated and the other had regular cycles of catatonic symptoms. Of the other 4 men, all were hallucinated. These 9 women and 4 men had

one or more of the following symptoms - thought disorder, emotional blunting, autistic symptoms, primary and secondary delusions and disturbance of volition. None of the subjects had received phenothiazines for at least a year but 6 of the schizophrenic patients received haloperidol during this initial study.

Diet was a normal hospital diet, but the following were excluded since some might give rise to a colour on the chromatograms and others contain amines and acids, the excretion of which was investigated in the study reported in Section B :- coffee, chocolate, cocoa, bananas, pineapple juice, tomatoes, tomato juice, tomato soup and tomato sauce, baked beans, plums, prunes, curry, beetroot and red-coloured foods, red sweets and red drinks, cheese, broad beans, beef and yeast extract. Tea and tobacco were withdrawn to the limits of the patient's tolerance.

Patients spent at least 24 hours in a two-bedded ward where fluid intake and output could be supervised and a complete 24 hour urine sample ensured. Fluid intake was restricted to one litre/24 hours for the reason given in the following section.

Urine collection and extraction

Urine was transferred soon after voiding to a polythene bottle containing 6% sodium azide and stored at a temperature of -20°C. It was then allowed to thaw in the refrigerator at 4°C over the next 24 hr. and was extracted at the end of this period.

Since the only centrifuge available to me would allow of the extraction of not more than 500ml. urine, the patients' fluid intake was restricted to one litre/24 hr. so that one-third of the 24 hr. urine sample could be processed.

In order to check the efficiency of the extraction procedure, two extra patients were included in the series and 22.5µg and 30µg d.m.p.e. added to their urine samples so that the one-third aliquots extracted contained 7.5µg and 10µg.

All urines were extracted in ignorance of the clinical state of the donor. Likewise it was not known to which urine samples d.m.p.e. had been added. An independent witness aided interpretation of the chromatograms.

Although a greater proportion of d.m.p.e. can be extracted into chloroform from urine at pH 10.0 than at pH 9.0, there was at the time of the study no convincing evidence that the 'pink spot' material detected by various investigators was d.m.p.e. Since Friedhoff and Van Winkle extracted their 'pink spot' precursors at pH 9.0 the same procedure was adopted in this study.

Estimation of urinary creatinine

Many authors allude to the quantity of urine extracted in terms of its creatinine content. One of the reasons for this seems to be that the 24 hr. urinary creatinine is considered to be constant for any one individual and use of this as a reference standard is preferable to a given proportion of a 24 hr. sample since this sample may be incomplete. However there is good evidence (Pscheidt et al 1966; Paterson 1967; Scott and Hurley 1968) that the 24 hr. creatinine excretion varies considerably from day to day in the case of individuals and there thus seemed to be little advantage in using this parameter. Nevertheless in order to be able to compare the results with those of others if this seemed necessary, urinary creatinine was estimated by Biggs and Cooper's modification (1961)

of the method of Folin (1914). This depended upon the reaction of creatinine with alkaline picrate and measurement of the optical density of the product.

Results

The quality of the chromatograms was variable. There seemed to be two reasons for this. Firstly, as anticipated, chromatography of an extract derived from one third of a 24 hr. sample overloaded the paper in that not only did pigment retard the migration of substances but so also did the considerable quantities of the basic materials present in the extract. Secondly, a difficulty met with in the preliminary study of methodology but not so far referred to was encountered again in this investigation. On some occasions reduction of the extract to damp dryness by nitrogen proved difficult in that a non-volatile viscous residue remained and application of this residue to chromatography paper resulted in a greasy patch on the paper and a streaked, retarded chromatogram. This phenomenon did not have an obvious explanation initially but was later discovered to be produced by the presence in the extract of silicone oil - used to lubricate the sleeve of the rotary flask - which had dissolved in the chloroform vapour and run back into the vessel with the condensed solvent (see p. 35).

Despite the poor quality of some of the chromatograms, the calculated risk was probably warranted because, of the forty patients investigated, eleven excreted pink spot material. That the accurate R_F value of the substances could not be determined was of no great consequence as such data could be obtained in a later study.

Recovery of d.m.p.e. added to urine One pink spot, due to introduced d.m.p.e. was detected. This was present in the urine to which 30µg, i.e. 10µg in the sample extracted, had been added. The other urine extract containing exogenous d.m.p.e. was not identified. The reason for this was that the extract in question yielded the most disappointing chromatogram of the series. It was pointed out at the time of viewing this chromatogram that both endogenous pink spot material and added d.m.p.e. would probably not be detectable.

Pink spot material detected The behaviour of a substance during chromatography is described by reference to the R_F value, i.e. the distance travelled by the substance as numerator compared with the extent of migration of the developing solvent as denominator. The R_F value of a substance in a urine extract will approximate to that of the compound in pure solution only when a small quantity of the substance and other materials is present. When greater amounts are present the various substances will interfere with the migration of the others, usually promoting a shorter chromatographic passage, although on occasion some materials will travel further than when chromatographed alone. When considerable retardation occurs, reference to R_F values becomes meaningless and resort to R_X values is preferable. This index compares the distances travelled by the material in question and another component of the extract. Since the degree of retardation of each substance will be similar, R_X values for matter detected on different chromatograms can be compared. The reference compound chosen in this study was urea, a constant component in urine extracts. The pink spot material and added d.m.p.e. migrated further than did urea.

Two groups of pink spot producing compounds were detected, those which travelled just a little further than urea and those which moved over a considerably greater distance.

i) Principal pink spot This was observed more frequently than any other. The R_{urea} was 1.05-1.15. Of the ten male schizophrenics, three had this chromatographic pink spot as did four of the ten females. Three of the ten male controls and one of the ten female controls also had this spot on their chromatograms. The positive male controls were diagnosed as suffering from G.P.I., frontal lobe injury and subnormality but there were as many "brain damaged" negative controls. The female suffered from depression.

ii) Other pink spots An orange pink (peach) spot preceded by ninhydrin blue, R_{urea} 1.42-1.52, was present on the chromatograms of one female schizophrenic and two female controls. The chromatograms of the psychotic patient also contained a 'principal pink spot'.

A reddish-pink spot which gave brown with ninhydrin, R_{urea} 1.52-1.55, was observed on the chromatogram of one female schizophrenic and one male control. A 'principal pink spot' was also noted on the chromatogram of the former subject.

Relationship of excretion of pink spot material and drug therapy

Table 3 lists treatment received by patients and 'pink spot' status. It can be seen that the administration of drugs bore no relation to the substances detected.

Further investigations of the 'pink spot' material excreted by the first two subjects screened

By the exercise of serendipity the first two urine samples of the forty

TABLE 3 Drug therapy of the subjects of the screening survey

Subject	Sex	Clinical State S - schizophrenic C - control	Drug Treatment (other than hypnotics)	Pink spot status ✳ Principal	† Other
1	F	S	Haloperidol	+	
2	F	S	Haloperidol	+	
8	F	S	Nil	+	+
30	F	S	Nil	+	+
13	M	S	Nil	+	
14	M	S	Nil	+	
21	M	S	Reserpine	+	
5	F	S	Haloperidol		
6	F	S	Nil		
7	F	S	Nil		
42	F	S	Nil		
43	F	S	Thioridazine		
45	F	S	Nil		
10	M	S	Haloperidol		
12	M	S	Haloperidol		
16	M	S	Nil		
18	M	S	Haloperidol		
22	M	S	Nil		
24	M	S	Protryptiline		
28	M	S	Phenobarbitone		
9	F	C	Nil	+	+
36	M	C	Nil	+	
37	M	C	Anticonvulsants	+	
38	M	C	Nil	+	
4	F	C	Amitryptiline		
44	F	C	Nil		
11	F	C	Lithium carbonate		
15	F	C	Amitryptiline		
17	F	C	Nil		
32	F	C	Nil		
33	F	C	Nil		
34	F	C	Nil		
35	F	C	Imipramine		
19	M	C	Nil		
23	M	C	Nil		
25	M	C	Nil		
27	M	C	Anticonvulsants		
29	M	C	Benzhexol		
40	M	C	Disulfiram		
41	M	C	Nil		

✳ As defined on page 30.

† Any of those so defined on page 30.

two examined contained pink spot material. As I had arranged with Dr. A.R. Somerville of I.C.I. Ltd. that I would, if possible, supply him with 'pink spot positive' urine, for purposes of identifying the material, his facilities being more extensive than mine, the opportunity was taken of retaining the two patients in the ward and storing at -20°C all urine collected during a period of one week. One tenth of every urine sample from each patient was stored in a separate bottle for each subject, the other nine tenths being collected in several bottles. Once 400ml. from each patient had accumulated in the bottles containing one tenth of each sample, these two 400ml. aliquots were extracted. No chromatographic pink spots were detected. In order to determine whether the pink spot material did not survive storage for more than a day or so, a freshly collected sample from each patient was extracted. These samples also failed to yield a positive result. The next step was extraction of an aliquot of one of the original 24 hr. samples which had contained the pink spot substance. This sample had been thawed out twice before this final extraction. Again a negative result was obtained. The conclusions drawn were that excretion of the substance was not constant and that the material did not survive prolonged storage. An alternative explanation was that the original observations had been invalid.

Within a few days of the first chromatographic evidence of excretion of pink spot matter, aliquots of the same 24 hr. samples were extracted and submitted to high voltage electrophoresis. I had gained experience of this technique during a three week period spent in Dr. Somerville's laboratory. A Locarte 5KV instrument was used. A one hour run at 3KV, 8 pounds per sq.in. on Whatman No.1 paper in 0.2M phosphate buffer pH 6.2 was undertaken. The extracts were divided into five sixths and

one sixth and applied to the paper as separate samples. When treated with the 'pink spot' reagents, the electrophoretograms of the five-sixths aliquots from each patient's extract yielded a pink zone which stretched a little ahead of and behind marker d.m.p.e. The pink spot material detected by Bell and Somerville (1966a) had behaved in this manner. The electrophoretograms of the one-sixth aliquots developed for d.m.p.e. fluorescence did not contain this amine in detectable amounts.

This latter investigation not only aided in identification of the pink spot substance but also verified the initial finding of the material upon which the subsequent failure to detect it in stored urine might have cast doubt.

Pink pigment extracted by chloroform The investigation had to be interrupted in its early stages when it was noted that extraction at pH2 of the third subject's urine sample yielded pink chloroform. This was found to occur in the case of some urine samples but not others. However it was also noted that a urine sample yielded this pink coloured chloroform on only some occasions. Therefore a laboratory artefact was suspected. The following observations were made. The pink substance extracted at pH2 was not obtained at pH6. Thorough cleaning by hot chromic acid of glassware and polythene bottles, the substitution of glass for polythene collection bottles, of tap for distilled, deionised water, of sulphuric acid for hydrochloric acid for pH adjustment, of dichloroethane for chloroform, and addition of ascorbic acid to hydrochloric acid did not prevent the extraction of pink matter. However the colour disappeared on shaking the chloroform with a reducing agent, sodium metabisulphite.

The urine of a biochemist and my children contained this pink pigment or its precursor. This observation appeared to exclude hospital diet as a source. On a subsequent occasion when my children were receiving oral penicillin, the chloroform extract of their urine was not pigmented.

These findings indicated that the substance responsible for the colour was an oxidising agent but its true nature and origin were not determined. Dietary and intestinal bacterial sources were considered possible. Once it had been ascertained that d.m.p.e. recovery was in no way affected by the presence of this material and that a laboratory artefact was not involved, the matter was not pursued further. The phenomenon persisted throughout the investigation but not in the case of every urine sample.

SUB-SECTION III

FURTHER IMPROVEMENT AND APPLICATION OF THE EXTRACTION PROCEDURE AND EXAMINATION OF AN ADDITIONAL EXTRACTION METHOD

In order to facilitate achievement of the final aim of the entire study i.e. to assess the significance of excretion of pink spot material and to determine, if possible, the nature of this substance, it was evident that the extraction procedure would have to be further modified. At the time of the screening study Boulton et al (1967) announced that a pink spot compound obtained from the urine of patients with schizophrenia and Parkinson's disease was p-tyramine. The conduct of the investigations had perforce to encompass examination of this claim. The work performed to these ends was undertaken both prior to and during the final investigation of patients but is more conveniently considered here in its entirety.

Improvement of the extraction procedure

The chief requirement for improving chromatograms was already known. Overloading of the paper with pigment and other solutes retarded migration of constituents of the final extract. Separate application to the paper of aliquots of extract equivalent to one-quarter and one-eighth of the 24 hr. urine sample effected satisfactory improvement.

Further to overcome this problem, it was decided to apply the extract to a strip of paper rather than to one point. However, it was realised that this procedure might so diminish the concentration - as opposed to quantity - of pink spot material present on the developed chromatogram as to impair visualisation. Application of this method in the final study would require to be cautious.

The other problem affecting chromatography i.e. the appearance of a non-volatile viscous residue in the extract and a greasy stain on the paper was fortunately solved. Use of the rotary evaporator required the lubrication of a rotating sleeve with a silicone oil which, the manufacturers

stated, was insoluble in most common organic solvents. I had earlier considered it possible that the lubricant might run back into the flask containing the chloroform extract but I finally realised and demonstrated that the silicone oil was dissolved in the chloroform distilling from the vessel and that this chloroform, condensing prematurely, then ran back into the container carrying the oil in solution. On reduction in volume of the extract by a jet of nitrogen, the chloroform evaporated, leaving the non-volatile viscous silicone oil behind. Substitution of glycerol, which proved not to dissolve in chloroform, for the commercial "insoluble" lubricant gave the desired result.

R_F Values

As the R_F of the frontal region of a spot on the chromatograms was found to be more useful than the R_F of the centre in that any tailing decreased the latter value, the rather unorthodox procedure of referring to the R_F values of substances as those of the fronts of the spots was adopted.

Efficiency of the solvent procedure for extraction of compounds known to give the pink spot reaction

Determination of the nature of the pink spot precursors detected would depend, inter alia, upon a knowledge of whether or not known pink spot producing compounds were extracted by the solvent procedure.

In a paper describing the development of the pink spot reaction Friedhoff and Van Winkle (1963b) listed the following substances as giving a positive reaction - β - phenylethylamine, tyramine, dopamine, 4 - hydroxy - 3 - methoxyphenylethylamine, 3,4 - dimethoxyphenylethylamine (d.m.p.e.), mescaline, amphetamine (weak positive), N-methyl-β-phenylethylamine (weak

positive), phenylalanine, 3,5-diiodotyrosine, and 3,4-dihydroxy-phenylalanine. Perry et al (1967) reported that monoacetyl cadaverine and monopropionyl cadaverine both responded to the reagents. As these substances are aliphatic amines, the claim of Friedhoff and Van Winkle that only aromatic compounds react was not upheld. The compound, 4-methoxyphenylethylamine, which was detected by Sen and McGeer (1964) in the urine of schizophrenic patients, also yielded a pink colour as did its isomer 3-methoxyphenylethylamine. The substance 3-hydroxy-4-methoxy-phenylethylamine, was also found in this study to be a reactor and doubtless there are others which were not tested.

The solvent procedure was 'scaled down' to allow the use of test tubes and the study of several compounds during one experiment. 40ml. of control urine and three 40ml. aliquots of the same urine with 50µg of three different reactors added, were extracted on each occasion. The following substances were tested - β-phenylethylamine, p-tyramine, dopamine, 4-hydroxy-3-methoxyphenylethylamine, 3-hydroxy-4-methoxyphenylethylamine, 3,4-dimethoxy-phenylethylamine, DL-phenylalanine, 3,5-diiodotyrosine, DL-dihydroxyphenylal-anine, monoacetyl cadaverine, monopropionyl cadaverine, 4-methoxyphenylethyl-amine and 3-methoxyphenylethylamine.

Of those substances added to urine the following were detected on the chromatograms - β-phenylethylamine, 4-hydroxy-3-methoxyphenylethylamine, 3-hydroxy-4-methoxyphenylethylamine, 3,4-dimethoxyphenylethylamine, 4-methoxyphenylethylamine and 3-methoxyphenylethylamine.

The absence of p-tyramine and the cadaverines from this list will be noted. Perry et al (1967) indicated that monopropionyl cadaverine was recovered poorly by the technique of Friedhoff and Van Winkle. On this

account the procedure was repeated, employing 500-1000µg of the cadaverine compounds. Distinct pink spots corresponding to both substances were obtained on the chromatograms. Since 5µg of either compound was required to yield an easily detectable spot, the recovery from urine on this basis was roughly 1%.

Examination of an ion-exchange method for the extraction of pink spot material

Several investigators (Takesada et al 1963; Faurbye and Pind 1964; Kuehl et al 1964) and also Boulton et al (1967) who identified pink spot matter as p-tyramine, employed an ion-exchange method as their urine extraction procedure prior to chromatographic separation of the components of the extract.

Method

The procedure was that of Kakimoto and Armstrong (1962) modified by Takesada et al (1963).

In order to remove contaminating substances the ion-exchange resin AG-50W-X 2, H+ form, 100-200 mesh was conditioned by treating successively with acetone, 2N sodium hydroxide, 2N hydrochloric acid and 1N ammonium hydroxide in 65% ethanol, water washes being interposed at each stage. The resin was stored in water and poured as a slurry into glass columns when required. The resin employed by Kakimoto and Armstrong was packed to a height of 2.5cm. in a column of 1cm. diameter. Since the columns immediately available in this study had an internal diameter of 0.6cm. the resin was allowed to settle (not packed) to a height of 7cm.

For these initial experiments 50ml. urine were employed. After filtration and adjustment to pH 5.0 (glass electrode) the urine was allowed to

run through the resin at an approximate rate of 20ml./hr. This was followed by 10ml. water, 20ml. 0.1N sodium acetate and 10ml. water at the same flow rate. This washing was designed to remove neutral substances and most of the aliphatic bases. Elution of the phenolic amines was effected with 10ml. 1N ammonium hydroxide in 65% ethanol at a flow rate of 15ml./hr. Following this elution Kakimoto and Armstrong reduced the samples to dryness and chromatographed the dissolved residue whereas Takesada et al dissolved the dried residue in 1ml. 0.1N sodium borate buffer pH9 and after shaking the buffer with 5ml. chloroform reduced the latter to dryness, dissolved this residue in ethanol and chromatographed it.

An attempt to reduce the eluate to damp dryness under a jet of nitrogen was made but owing to the water content this procedure proved impractical. While realising that exposure to oxidation by atmospheric oxygen would result, the only feasible alternative seemed to be to follow the method of the original authors. The eluate was reduced to dryness in an atmosphere of nitrogen in a rotary evaporator at 32°C. The residue was either dissolved in methanol-ascorbic acid solution for chromatography or was dissolved in 1ml. 0.1M glycine-sodium hydroxide buffer pH9.0 and extracted with 5ml. chloroform. Following reduction to dryness under a jet of nitrogen the residue from the chloroform extract was dissolved in methanol-ascorbic acid solution for chromatography.

Results

The chromatograms derived from extracts treated according to Kakimoto and Armstrong differed in two major respects from those resulting from inclusion of the buffer step of Takesada et al. Even extracts of 5ml. urine were heavily pigmented and streaking of this pigment to the origin occurred

unless the buffer was employed. Pigment posed no problem whatsoever under the latter circumstance and this was presumably the reason for the modification adopted by Takesada et al. All urines tested - from two children and ten nurses - contained pink spot material. There was a considerable amount of this present in the non-buffer washed extract as evidenced by intense colour. This pink spot substance exhibited considerable tailing (streaking) but this effect was overcome by the use of the buffer step. A compact spot was obtained but it was evident that the greater proportion of the material was retained in the buffer.

That the pink spot precursor did not have its origin in the ion-exchange resin or the various solutions used, was proved by failure to detect a pink colour when water was substituted for urine. Such a precaution was necessary because Dr. A.R. Somerville (personal communication) had obtained pink spot matter, the source of which was impurity in his resin.

The R_F of the material was identical with that of p-tyramine.

Application of the ion-exchange method to extraction of various pink spot precursors

It had been intended to estimate the recovery of all of the pink spot substances as for the solvent procedure. However the presence in the urine of so much of the substance which behaved like p-tyramine necessitated the addition of unduly large amounts of other compounds. The result of such addition was poor chromatographic separation of the endogenous and exogenous reactors caused by overlapping of the spots.

The recovery of monoacetyl cadaverine was examined despite this difficulty. In particular the reported effect of the sodium acetate

and water washes i.e. displacement of most aliphatic amines was investigated, monoacetyl cadaverine being such a substance. Two pairs of columns were developed. Over one of each pair was passed urine to which 20µg of the amine had been added and over the other a urine sample containing 300µg monoacetyl cadaverine. One pair was developed according to the established method but water was substituted for sodium acetate during treatment of the other. The base was recovered only from those columns to which sodium acetate had not been applied. Thus monoacetyl cadaverine was eluted by the solution from the other two columns.

Para-tyramine. Detection and estimation, urinary excretion and extraction from urine

Two preliminary observations have already been made concerning p-tyramine. It could not be recovered as a pink spot when 40ml. urine containing 50µg of added amine were extracted by the solvent procedure whereas untreated urine samples processed by the ion-exchange method yielded a pink spot precursor having an R_F identical with that of p-tyramine.

a) Detection and measurement of p-tyramine by development of a fluorophor on paper

Boulton (personal communication) employed a fluorimetric method for the detection and estimation of p-tyramine on paper chromatograms. The method was a modification of that described by Udenfriend (1962), intended for assay of p-tyramine in solution. The chromatogram was dipped through 25ml. of 9vol. of 0.1% ∝-nitroso-βnaphthol in ethanol - 1vol. of conc. nitric acid containing 0.13ml. 2.5% sodium nitrite, drained for a few minutes and heated at 130°C for 15 min. A fluorophor was then visible under ultra-violet light. Boulton employed a

paper chromatogram strip scanning device to measure the fluorescence characteristics of p-tyramine present on the paper. This method, with the omission of the last step, was examined. A faint gold-yellow fluorophor was produced by treatment of paper containing 1µg p-tyramine and fluorescence was proportionally greater with larger amounts. However, even 5µg did not yield a very marked fluorescence. Quantities of less than 0.5µg were not discernible. It seemed likely that the presence of more than 5µg in a urinary extract would be necessary for visualisation. On several occasions chromatograms of urinary extracts obtained by the ion-exchange procedure were developed according to this method. A number of yellow fluorophors, present at various situations from origin to solvent front were detected. As several were observed near the position adopted by marker p-tyramine, it would have been difficult to decide which to choose for further examination. As a paper scanning device was not available, this method was rejected as unsuitable for the purpose of this study.

b) Detection and measurement of p-tyramine by development of a fluorophor in solution

The method of Udenfriend (1962) already referred to has been widely used for the estimation of urinary p-tyramine in the eluates from ion-exchange columns. Absence of interfering substances, implied by its use for such purposes, rendered it a likely method for identifying p-tyramine eluted from a chromatogram.

i. Method

Reagents required were 0.1% α nitroso-β-naphthol in ethanol and nitric acid reagent i.e. 10ml. conc. nitric acid to which had been added 1.2ml. 2.5% sodium nitrite and water to 60ml. To a 3ml. sample were

added 1.5ml. α-nitroso-β-naphthol and 1.5ml. nitric acid reagent. After mixing the samples were heated in stoppered test-tubes in a water bath at 55°C for 30min. The unreacted α-nitroso-β-naphthol was then removed by shaking with 7ml. dichloroethane. The fluorescence of the supernatant at 565mμ when excited at 465mμ was determined. Employing a Farrand spectrophotofluorimeter in this study the maxima were 465/560mμ (uncorrected) and when an Aminco-Bowman was used the maxima were 465/570mμ (uncorrected).

The nature of the nitroso-naphthol product of tyramine is not known.

ii. Specificity

Tyrosine, p-hydroxyphenylacetic acid, p-hydroxyphenylpyruvic acid, homogentisic acid, p-hydroxyphenylethanolamine and N-methyl-p-hydroxyphenylethanolamine all give identical fluorescent products (Udenfriend 1962). However the acid products, even if present, are removed by dichloroethane and there are only small amounts of the free ethanolamines in urine. Tyrosine would not be present in the extracts employed in this study.

It was also ascertained that none of the following compounds formed a similar fluorophor - m-tyramine, β-phenylethylamine, 3-methoxyphenylethylamine, 4-methoxyphenylethylamine, 3-hydroxy-4-methoxyphenylethylamine, 4-hydroxy-3-methoxyphenylethylamine, tryptamine, monoacetyl cadaverine and monopropionyl cadaverine.

iii. Sensitivity

As little as 0.1-0.2μg can be detected in a 3ml. sample.

iv. Linearity

Udenfriend states that extraction of 10ml. urine samples allows accurate measurement of 30μg to approximately 2,000 μg p-tyramine/litre. This corresponds to the presence in the final sample assayed for fluor-

escence of 0.3-20µg. This was confirmed for p-tyramine in pure solution in the range 0.1-15µg (Fig. 4).

v. Method applied to estimation of p-tyramine eluted from paper.

In order to diminish interference from urinary pigment and other constituents and to ensure accurate R_F values, the final extracts intended for chromatography obtained from both solvent and ion-exchange procedures were streaked along a line at the base of a 3cm. strip of Whatman No.1 paper. On every occasion a 1.0µg standard of p-tyramine was applied to a similar strip. Following chromatographic development in n-butanol-acetic acid - water the strips were divided into 2cm. segments and each segment immersed in 3ml. 0.1N hydrochloric acid in a glass-stoppered test-tube. After standing for 1 hr. at 4°C and 30 min. at room temperature, the paper strips were pulled out of the acid, allowed to drain, and discarded. The p-tyramine content of each 3ml. sample was then determined.

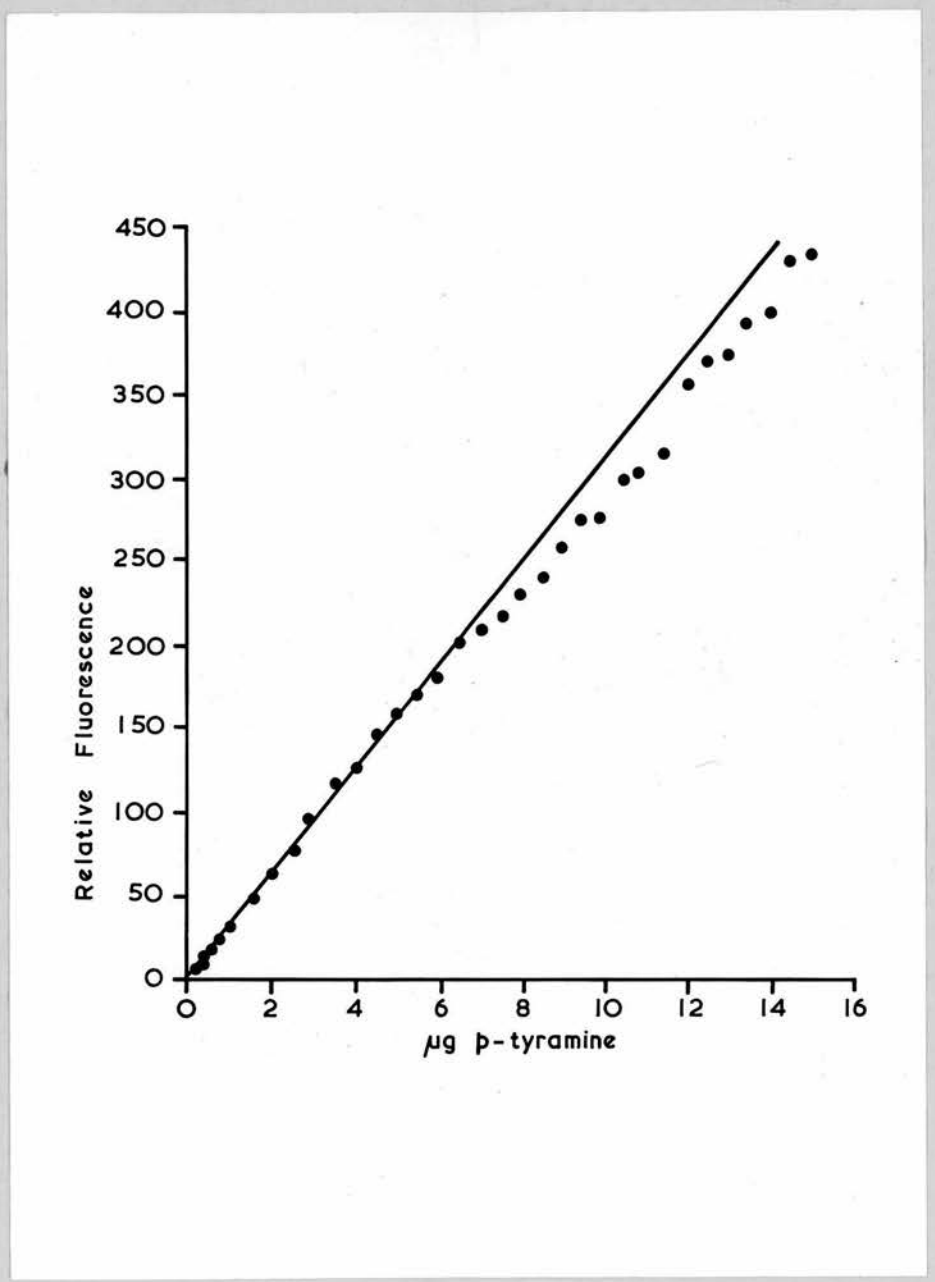
Comparison of the fluorescence produced by the 1.0µg standard eluted from paper and a 1.0µg standard in pure solution indicated that the efficiency of elution was 80-90%. Fluorescence at the appropriate excitation and fluorescence maxima was detected in one, two or at most three adjacent segments from the chromatographic strips derived from urine extracts. The R_F values obtained from urine extracts varied between 0.58 and 0.66, the range for marker p-tyramine being 0.6-0.66.

c) Urinary excretion of p-tyramine by schizophrenic subjects

To support their contention that the pink spot material they had detected was p-tyramine, Boulton et al (1967) reported excretion of "abnormally large amounts of urinary p-tyramine" by a schizophrenic and four patients with Parkinsonism. In a urine sample from a schizophrenic

Fig. 4

Calibration curve for p-tyramine estimated by the method of Udenfriend



patient, who excreted a pink spot precursor, they measured 1,289µg/1.1g creatinine and in another 'pink spot' urine, pooled from the patients with Parkinson's disease, 2,211µg/1.1g creatinine.

The 24hr. urinary p-tyramine of each of nine of the schizophrenic patients who had been studied in the screening survey was determined. Four of these subjects had excreted detectable pink spot material of the type described as principal in that survey and five had not. Additionally the tryptamine and 5-hydroxytryptamine (5-HT) content of each urine sample was estimated. The methods employed are described in Section B. 24hr. urinary creatinine was also measured.

The results are given in Tables 4 and 5. Also included in the Tables are the 24hr. urine volume and creatinine during the screening survey. Whereas fluid intake had been restricted to one litre/24hr. on that occasion there was no limitation on fluid intake during the period of urine collection for p-tyramine estimation.

For purposes of comparison 24hr. urinary p-tyramine values for eight healthy subjects are given in Table 6. These had been determined on a previous occasion by a member of the M.R.C. Unit for Research in Brain Metabolism.

It was decided that the samples were too small to justify the application of statistical techniques. However, two trends can be noted. The mean 24hr. urinary p-tyramine of pink spot substance excretors was greater than that of the non-excretors but the mean values were no greater than that of normal subjects. The mean 24hr. urine volume of excretors of pink spot material was greater than that of the non-excretors. The difference was noticeable when data pertaining to the period of unrestricted

TABLE 4

Urine volume, amines and creatinine of 9 schizophrenic subjects

Sex, Code No. age.	Pink spot status	Fluid intake unrestricted					Fluid intake restricted	
		Tyramine µg/24hr.	Tryptamine µg/24hr.	5HT µg/24hr.	Urine vol. ml./24hr.	Creatinine g./24hr.	Urine vol. ml./24hr.	Creatinine g./24hr.
F 1 (59)	+	330	120	40	1900	0.9	1250	0.9
F 2 (37)	+	300	60	150	2960	1.5	1030	1.1
M 14 (48)	+	150	130	110	4560	1.5	1150	1.1
M 21 (57)	+	410	170	350	3140	1.8	1300	1.2
M 10 (58)	-	160	110	70	1790	1.3	980	0.9
M 12 (31)	-	210	170	150	2970	2.0	1450	1.1
M 22 (58)	-	210	80	190	1410	1.4	840	1.3
M 24 (47)	-	130	40	180	1240	1.0	1030	1.0
M 28 (59)	-	280	70	180	850	1.3	440	0.6

TABLE 5

Mean urine volume, amines and creatinine of 9 schizophrenic subjects
and
standard errors of the means

	Fluid intake unrestricted					Fluid intake restricted	
	Tyramine µg/24hr.	Tryptamine µg/24hr.	5HT µg/24hr.	Urine vol. ml./24hr.	Creatinine g./24hr.	Urine vol. ml./24hr.	Creatinine g./24hr.
Pink spot subjects +	313±48	134±21	178±58	3100±470	1.41±0·16	1610±220	1.12±0·06
Pink spot subjects -	197±23	95±20	153±20	1650±330	1.38±0·15	950±150	1.0±0·11

TABLE 6

Urine tyramine and urine volume of 8 healthy subjects

(Dr. D. Eccleston, M.R.C. Unit for Research in Brain Metabolism-unpublished data)

Subject	Tyramine µg/24hr.	Urine vol. ml./24hr.
1	290	810
2	260	810
3	420	1780
4	340	530
5	600	1200
6	330	860
7	630	1530
8	510	2030
Mean values	422.5	1193.7

fluid intake were considered. However there was a not inconsiderable overlap between the two groups of 24hr. volume values.

No meaningful explanation of all the data could be found and it is likely that the small numbers involved were responsible for apparent differences. However it was considered that a further study of p-tyramine excretion was warranted and that the possibility of a relationship between excretion of pink spot material and urine volume was worth entertaining.

The other point of note was that the p-tyramine values were considerably lower than those quoted by Boulton et al. However these investigators employed the method of Kakimoto and Armstrong (1962) for concentration of urinary phenolic amines. Although this method has already been outlined (p. 38), the description of one step in the procedure was omitted as it was not incorporated in the preliminary work. Kakimoto and Armstrong collected the column effluent and hydrolysed it to release conjugated amines. The hydrolysate was then applied to the ion-exchange resin and the resin developed as for the unhydrolysed sample. Thus the p-tyramine data quoted by Boulton et al referred to the total quantity of the amine whereas these obtained in this study did not include values for the conjugated form. Therefore in order to confirm or refute the findings of these authors, urine would require to be hydrolysed. Methods and results for this procedure are discussed in Section B.

d) Recovery of p-tyramine by the solvent extraction procedure

In the section dealing with the efficiency of the solvent procedure for extraction of known pink spot substances, it was noted that a chromatographic pink spot was not obtained when 40ml. urine containing 50µg added p-tyramine were extracted. Boulton et al detected 1,289µg p-tyramine/1.1g

creatinine in the urine of a schizophrenic patient who had excreted pink spot material identified as p-tyramine. If one assumes the urine volume to have been about 1,100ml. then 40ml. urine would have contained somewhat less than 50µg. Thus by chance the concentration I had chosen for purposes of experiment tallied with that detected by Boulton et al. If the failure to recover this amount as a pink spot was confirmed then doubt would be cast on the claims of those workers.

Boulton et al (1967) implied that p-tyramine was unstable under alkaline conditions. The extraction of 100µg p-tyramine added to 40ml. urine was undertaken, care being taken to stir the urine constantly during addition of sodium hydroxide to avoid high local concentrations of alkali and to extract with chloroform immediately following adjustment to pH9.0. Again no pink spot was obtained.

On several subsequent occasions extraction of 300ml. urine containing 750µg-1,000µg yielded similar negative results. The presence of p-tyramine in the extracts of those treated urines was sought not only as a pink spot on the chromatogram but also as a nitrosonaphthol fluorophor, using the technique of elution from paper strips.

e) Partition coefficient of p-tyramine between chloroform and aqueous phase

In order to discover why p-tyramine could not be recovered from urine in detectable amounts by the solvent procedure, the partition coefficient of the amine between chloroform and buffer pH9.0 was determined.

Duplicate samples of 10ml.0.2M glycine-sodium hydroxide buffer pH9.0 containing 10µg p-tyramine were shaken for 4min. with 10ml. chloroform. 9ml. of the chloroform from each 10ml. was shaken with 10ml.0.1N hydrochloric acid pH1.0 for 4min. A p-tyramine-free buffer sample was similarly

treated.

3ml. aliquots of the three buffer samples were taken after chloroform extraction and 3ml. of each of the hydrochloric acid back-extraction samples were removed. Other samples were 3ml. water, four p-tyramine standards of 0.5, 1.0, 2.0 and 5.0µg in 3ml. water, 3ml. of the buffer, 3ml. of the buffer containing 1µg p-tyramine to test for quenching, and duplicate samples of 3µg p-tyramine in 3ml. buffer. The p-tyramine content of all samples was assayed by the fluorescence procedure of Udenfriend (1962).

Result

The relative fluorescence for the various samples are recorded in Table 7. It will be seen that virtually no p-tyramine was extracted into the chloroform. From the calibration curve (not shown) constructed from the values for the standards and allowing for the fact that only 9ml. of the 10ml. chloroform extracts were back-extracted with acid, it was calculated that the 10ml. acid used to extract the chloroform contained 0.18µg of the amine. As can be seen from the table, the method was not sufficiently sensitive to indicate that this small amount had been removed from the buffer by extraction. As a very small quantity of p-tyramine remained in the chloroform after acid extraction, the partition coefficient was slightly greater than $\frac{0.18}{9.82}$. Rounded off to realistic figures the partition coefficient was therefore of the order 0.02.

This figure was corroborated by the calculation made from the proportion of p-tyramine in the ion-exchange column eluate extracted into chloroform and referred to under f) ii below. The ratio determined from that evidence was also 0.02.

f) Pink spot material obtained from urine by ion-exchange. Application of the nitroso-naphthol reaction for purposes of identification

TABLE 7

p-tyramine content of samples used for determination of partition coefficient $\frac{\text{chloroform}}{\text{buffer}}$

Sample		Relative fluorescence
Standards	0.5µg	21
	1.0µg	38
	2.0µg	73
	5.0µg	193
Sample to test for quenching:-	Buffer containing 1µg	40
3ml. buffer containing 3µg	Duplicate	122
	Duplicate	125
3ml. from 10ml. buffer after chloroform extraction	Duplicate	122
	Duplicate	122
3ml. from 10ml. HCl	Duplicate	2
	Duplicate	1

The appropriate blank readings have been subtracted.

In the section dealing with the use of ion-exchange column chromatography it was noted that the urine of each of twelve healthy subjects contained material which gave a pink spot reaction and had an R_F identical to that of p-tyramine.

i. Further identification as p-tyramine Chromatography of the final ion-exchange extract and elution of the segments from the chromatogram as already described always yielded a nitroso-naphthol fluorophor in one, two or at most three adjacent segments at the R_F location of p-tyramine.

ii. Effect of the modification of Takesada et al on p-tyramine recovery

It was noted previously that the interposition of solution in buffer and extraction into chloroform between the stages of drying the column eluate and chromatography produced a compact pink spot but drastically reduced the amount of the responsible material recovered from the eluate. This was confirmed by application of strip elution and fluorophor formation. It was found that the buffer-chloroform step reduced the quantity of p-tyramine recovered ninefold. Thus 90% remained in the buffer, only 10% being extracted by the chloroform. Since 5ml. chloroform and 1ml. buffer were employed, 1ml. chloroform would have removed 2%. On this basis the partition coefficient $\frac{\text{chloroform}}{\text{buffer}}$ at pH9.0 was 0.02.

iii. Apparent discrepancy between the amounts of p-tyramine detected as pink spot and nitroso-naphthol fluorophor

Parallel extracts, obtained by ion-exchange, were developed for pink spot and nitroso-naphthol fluorescence. In every instance, employing urine samples from different subjects, it appeared that the quantity of p-tyramine present on the chromatogram as estimated fluorimetrically was not sufficient to account for the pink spot obtained. Thus 1-2μg were detected by fluorimetric assay whereas the intensity of the pink spot

indicated the presence of 3-5µg.

Two explanations for this anomaly were considered. The fluorimetric assay may have given falsely low values owing to quenching. This possibility was excluded by demonstrating that 1.0µg p-tyramine produced the same amount of fluorescence when added to either pure solution or a urinary extract. Alternatively, the pink spot was not produced solely by p-tyramine. This would have required the presence of another pink spot substance with the same R_F or of another material which, while not giving a pink spot reaction, enhanced the pink colour i.e. produced a bathychromic effect.

As will be seen in Sub-section IV another pink spot reactor was present.

iv. Release of p-tyramine by acid hydrolysis of urine It has been noted that Boulton et al (1967) estimated greater p-tyramine content of 24hr. urine samples than was found initially in this study because they hydrolysed the urine to effect the release of conjugated p-tyramine. The effect of acid hydrolysis was therefore examined. Urine was adjusted to pH1.0 or 2.0 (glass electrode) with conc. hydrochloric acid and boiled for 30min. in a flask fitted with a reflux condenser. Prior to heating, nitrogen was vigorously bubbled through the urine and this was continued during boiling in order to reduce exposure to oxidation. After boiling, the urine was cooled and restored to the original volume with distilled water.

Employing equal aliquots from the same sample of urine, it was demonstrated that hydrolysis at pH2.0 increased the estimated p-tyramine content by 50% and at pH1.0 by over 100%.

This finding was applied in two ways. Firstly, in the detailed

study of patients described in Sub-section IV, portions of urine were hydrolysed in order to determine whether p-tyramine could be recovered by the solvent procedure as a pink spot. Secondly, as discussed in Section B, the procedure was employed for estimation of 24hr. urinary p-tyramine.

Summary of pink spot, nitroso-naphthol and glycine/formaldehyde reactions of compounds of interest

In Table 8 are listed the ninhydrin and Ehrlich colours, the reaction with nitroso-naphthol-nitric acid reagent on paper and in solution, the reaction with formaldehyde (Bell and Somerville 1966b) and the R_F in n-butanol-acetic acid - water of compounds which required to be considered when determining the nature of the pink spot substances.

TABLE 8 Reactions of pink spot compounds and tryptamine

Compound	Ninhydrin colour	Ehrlich colour	R_F in n-butanol-acetic acid-water (front of spot)	Nitrosonaphthol Fluorescence		Glycine formaldehyde reaction
				On paper	In solution	
β-phenylethyl amine	Royal blue	Orange pink (peach)	0.73 - 0.77	-	-	-
3-methoxy phenylethyl amine	Blue green	Orange pink (peach)	0.76	Not tested	-	-
4-methoxy phenylethyl amine	Blue grey	Red pink	0.74	Not tested	-	-
3-hydroxy-4-methoxyphenyl ethylamine	Red brown	Red pink	0.60	Not tested	-	ice blue 360 mμ exc. 470 mμ fl.
4-hydroxy-3 methoxyphenyl ethylamine	Grey blue	Red pink	0.62	-	-	yellow 440 mμ exc. 520 mμ fl.
3,4-dimethoxy phenylethyl amine (d.m.p.e.)	Blue grey	Red pink	0.66 - 0.71	-	-	ice blue 360 mμ exc. 480 mμ fl.
p-tyramine	Blue grey	Red pink	0.60 - 0.66	+	Yellow 465 mμ exc. 560 mμ fl.	-
m-tyramine	Blue grey	Red pink	Not tested	Not tested	-	Not tested
Monoacetyl cadaverine	Blue violet	Salmon pink	0.56 - 0.60	Not tested	-	-
Monopropionyl cadaverine	Blue violet	Orange pink (peach)	0.69	Not tested	-	-
Tryptamine	Brown	Red brown or orange	0.68	Not tested	-	green-yellow

SUB-SECTION IV

APPLICATION OF THE TWO EXTRACTION PROCEDURES TO THE STUDY OF PINK SPOT MATERIAL EXCRETED BY PATIENTS AND HEALTHY CONTROLS

Purpose of the study

The object of this final investigation was the more detailed examination of excretion of pink spot material by subjects selected from the forty included in the screening survey and by healthy controls. The crucial question to be answered was whether or not excretion of pink spot precursors was in any way related to schizophrenia. The project was also designed to probe three other problems. As there were indications from the study described in Sub-section III that the pink spot compounds obtained by the two extraction procedures were not the same, substantiation was important, if only to help resolve the confusion reigning as a result of the already published reports. To date, only one report has compared the two methods and the authors (Pind and Faurbye 1966) concluded that the substances obtained by Friedhoff and Van Winkle (1962) and by Takesada et al (1963) were identical. Although a logical inference could not be drawn from the results of the investigation reported in Sub-section III concerning p-tyramine excretion [illegible] and urine volume [illegible], it seemed worthwhile to pursue an intuition that excretion of pink spot material could be correlated with urine volume. Linked with this aspect of the investigation was a determination of the dependence, if any, of excretion of pink spot matter on urinary pH. The reason for this interest in urinary pH was the documented evidence that some weak bases are excreted more efficiently under acid conditions (see Section B for a more detailed discussion of this subject). Finally it was deemed essential to essay identification of the material encountered although conclusions could only be tentative as the more refined techniques

such as infra-red and mass spectrography necessary for such delineation were not available.

Methods

Selection of Subjects

Eight patients from the original forty were chosen, four schizophrenics and four controls, two of each group having excreted, during the screening study, pink spot material described as "principal" in Sub-Section II. Three of the schizophrenics had active symptoms of schizophrenia as defined in the screening study section.

Each patient remained in a ward for four weeks where behaviour, diet and fluid balance could be observed. Diet was as in the initial study. As also in the screening study, no phenothiazines had been administered for at least one year. In order to reduce the likelihood of drug metabolites appearing on the chromatograms and because it has been reported to affect dopamine metabolism (Roos 1964; Bruno and Allegranza 1965; Andén et al 1966), haloperidol and, when possible, other drugs (exceptions being such as anticonvulsants) were withdrawn two weeks before investigation.

Three healthy young, volunteer nurses, one male and two female, subject to the same fluid and dietary restrictions but living in the ward for only the 24hr. of urine collection, were also subjects of this study.

Control of urinary volume and pH Three regimens were employed in random order for each subject and a fourth was used during the final week of each subject's stay in the ward. The reason for this latter was that to have sought pink spot precursors under all conditions would have rendered the study unduly long. Although excretion of acids and amines (see Section B)

was examined for four weeks, it was found possible to introduce a new subject into the ward for 'pink spot' investigation during the week of collection of alkaline urine i.e. the fourth week.

The first regimen consisted of a fluid intake of one litre per 24hr. for 48hr. and urine collection during the second 24hr. The second was as the first but during the 48hr. before and the 24hr. of the urine collection ammonium chloride 90mg. six times per day was taken by mouth. Because of the tendency of this drug to cause nausea and probable refusal by the patients to take it, this rather small dose was chosen. The urinary pH on this regimen was 5.2 to 5.7. The third regimen required a fluid intake of two litres per 24hr. for 48hr. and urine collection during the second 24hr. Subjects adhering to the fourth regimen took 2.5g. sodium bicarbonate every two waking hours over a 72hr. period during the final 48hr. of which fluid intake totalled two litres, urine being collected during the final 24hr. The urinary pH on this regimen was 7.9 to 8.4. The four urine samples from each subject were obtained at weekly intervals.

Analytical Methods

All urine samples were extracted in ignorance of the identity of the donor, the "blind" being broken only at the end of the study. The entire procedure occupied three days. Extracts from the dried column eluates and the chloroform obtained by reduction in volume by rotary evaporator of the solvent extracts were stored overnight at -20°C. On the following day the extracts were prepared for chromatography and development begun at midday. An advantage of this procedure was the 19-21hr. duration of paper chromatography and the greater separation of components of the extract achieved by the resulting 34-36cm. migration of the solvent.

1. Routine procedures

a) Solvent extraction procedure Three-eighths of the 24hr. urine sample was hydrolysed at pH1.0 as already described. This urine and a similar aliquot of untreated urine were processed by the solvent method. Portions equal to two-thirds and to one-third of each urinary extract were chromatographed in parallel. These final extracts were thus derived from one-quarter and one-eighth of the 24hr. urine sample.

i. Development for pink spot All of the above chromatographed extracts were developed for pink spot.

ii. Detection of p-tyramine Additionally the chloroform extract from hydrolysed urine equivalent to 50mg. creatinine was chromatographed and the p-tyramine content estimated by the fluorescence method.

iii. D.m.p.e. detection In the case of urine collected under conditions of forced diuresis (fluid intake 2 litres/24hr.), the chromatogram of an extract equivalent to one-eighth of the 24hr. sample (unhydrolysed) was developed for d.m.p.e. fluorescence.

b) Ion-exchange chromatography Two aliquots of 30-100ml. untreated urine and two similar aliquots of urine hydrolysed at pH1.0 were adjusted to pH5.0 (glass electrode) with conc. hydrochloric acid or 2.5N sodium hydroxide and extracted by the ion-exchange procedure. Volumes of urine employed were dictated by the volume remaining from the 24hr. collection. The dried eluate derived from one of the unhydrolysed aliquots of urine was dissolved in methanol-ascorbic acid solution while the other three dried eluates were dissolved in the buffer pH9.0 and extracted with chloroform.

Examination of extracts for d.m.p.e., p-tyramine and other pink spot substances

The extract of one aliquot of hydrolysed urine was chromatographed and assayed for p-tyramine content while the other three extracts were developed for the pink spot reaction.

As in the case of solvent extracts, d.m.p.e. was sought in those obtained by ion-exchange chromatography of the urine obtained during forced diuresis. For this purpose 100ml. unhydrolysed urine was applied to the resin.

2. Variations in procedure during the study

a) Omission of the buffer wash from the solvent procedure During the study, failure to detect p-tyramine in the solvent extracts might have been due to inclusion of the pH10.0 buffer wash. The partition coefficient would favour this interpretation. Later in the study it was decided to compare washed and unwashed extracts. Adoption of this procedure demonstrated that a considerable proportion of the pink spot material was also removed by the wash. Of the eight patients, only two were investigated without use of this refinement.

b) Extracts applied to paper by streaking When considering improvement of the extraction procedure in Sub-section III, it was decided that application of the extracts to chromatography paper by streaking rather than to a single point would be undertaken cautiously lest the pink spot material be spread over too wide an area to be detectable. During the study this was found to occur. Streak application was therefore reserved for the extract derived from one-quarter of the 24hr. urine sample (unhydrolysed) in order to obtain a reliable R_F value should a colour be obtained. The extract equivalent to one-eighth of the 24hr. urine sample (unhydrolysed) was applied as a spot.

3. Additional procedures

In addition to the above described procedures which were applied to the urine of all subjects, certain other investigations were undertaken, chiefly to elucidate the nature of the pink spot material.

a) Identity of the pink spot material obtained by the solvent procedure with d.m.p.e. or p-tyramine When pink spot material was detected by the solvent procedure the opportunity was taken to develop parallel extracts, obtained from one-eighth or one-quarter of the 24hr. sample, for pink spot and d.m.p.e. or p-tyramine.

b) The use of other solvent systems for paper chromatography The pink spot substances, obtained by the two different procedures, were chromatographed in solvent systems other than n-butanol-acetic acid - water (4:1:1 by vol.)

c) High voltage electrophoresis Various of the synthetic pink spot compounds and the pink spot material obtained from urine by the ion-exchange procedure were studied by high voltage electrophoresis in 0.2M phosphate buffer pH6.2.

d) The effect of oral administration of antibiotics on the excretion of pink spot substances Since a claim had been made that it was bacterial in origin, pink spot material was sought by the solvent procedure in the urine of one subject before and after antibiotics had been administered in order to eradicate the commensal organisms in his gut.

Results

The pink spot producing compounds obtained in the eluates from the ion-exchange resin were not the same as those obtained by solvent extraction.

a) Solvent extraction method Various pink spot compounds were observed on chromatograms of extracts obtained by the solvent-extraction procedure.

It is worth noting that "pink" covers a wide range of shades - p-tyramine and d.m.p.e. give reddish pinks, monoacetyl cadaverine a salmon pink, β-phenylethylamine, 3-methoxyphenylethylamine and monopropionyl cadaverine an orange pink or peach. Tryptamine gives either reddish-brown or orange, not pink. However, failure of separation from other isographic substances, in particular those giving rise to an orange colour, will result in a difference of shade produced by a compound in the urine extract and the same compound in pure solution. This applies to a much lesser extent to chromatograms of column eluates, there being fewer ninhydrin-positive compounds present.

Chromatographed extracts from hydrolysed urine contained so much pigment as to render interpretation difficult. The following findings therefore apply only to unhydrolysed urine.

i. Principal pink spot

A pink spot of greater frequency than any other detected by this method was obtained on the chromatograms and was probably the same as the principal pink spot in the initial survey. Presumably due to the presence of isographic material, no consistent ninhydrin colour preceded the pink which latter was a shade between the red-pink of tyramine and salmon pink of monoacetyl cadaverine. The R_F value in n-butanol-acetic-acid - water (4:1:1 by vol.) was 0.51 to 0.57.

Relation to urine volume The material yielding this pink spot was present in extracts of urine obtained from two schizophrenics and two controls under both conditions of fluid intake. In two schizophrenics and two controls in whose urine it was not detected on a restricted fluid regimen, forced diuresis effected its appearance. It was also present in

the urine of all three volunteer nurses on a restricted fluid regimen. The presence or absence of this chromatographic pink spot persisted during the two weeks when urine samples were collected under conditions of fluid restriction.

As it happened, the two non-schizophrenic patients who excreted pink spot material under conditions of restricted fluid intake during the screening survey did so only when fluid intake was increased in the course of this study and these two who had no detectable pink spot precursor in their urine during the first study excreted the material whether fluid intake was restricted or not during the second. For the schizophrenic patients the pattern of excretion was the same during the second as during the first investigation. The implications of these observations are several. Firstly, they do not affect the conclusion that if a subject did not excrete a detectable pink spot material, its detection could be effected by increasing the fluid intake and consequent urinary output. Secondly, the excretion of the substance varies with other unidentified influence(s). Thirdly, the number of subjects in a given sample who excreted a pink spot substance on a restricted fluid intake was greater than suspected from the results of the screening survey.

Effect of urinary pH Rendering the urine acid did not appear to affect the detectability of the pink spot material. As various factors influence pH dependence, no conclusions as to the chemical structure of the substance could be drawn.

Differentiation from p-tyramine That this pink spot was not p-tyramine was proved by the chromatographic separation in n-butanol-acetic-acid - water (4:1:1 by vol.) of the pink spot and p-tyramine hydrochloride (which migrates in an identical fashion to the free base) which was added to the extract

prior to chromatography and by the failure of an extract, similar to that yielding a pink spot, to reveal the presence of p-tyramine on a chromatogram by the fluorimetric method.

As no fluorimetric evidence was obtained for the presence of p-tyramine in extracts of untreated urine equivalent to one-eighth of the 24hr. sample similar quantities of extract from hydrolysed urine were analysed on several occasions. A difference in the fluorimetric scans for extracts buffer-washed and not buffer-washed was observed. A typical response from the p-tyramine fluorophor was a tall sharp peak at 560mμ. If a smaller quantity was present a low rounded curve was obtained but the maximum height was at 560mμ. In the case of an unwashed extract of hydrolysed urine there was an increase in fluorescence at 560mμ not only at the appropriate R_F location of p-tyramine but also beyond this position. However there was also increased fluorescence at other wavelengths in the region of 560mμ and the effect was of a broad plateau with no maximum fluorescence at 560mμ. It was possible that some of the fluorescence was attributable to small amounts of p-tyramine (less than 1μg) but certain that most, if not all, was due to other substances.

Moreover in the case of an extract of unhydrolysed urine equivalent to one-quarter of a 24hr. sample, a Zeiss spectrophotofluorimeter was employed. The separation of fluorescence peaks at wavelengths not much removed from each other achieved by this instrument is superior to that of the Farrand and Aminco-Bowman instruments. No p-tyramine was detected.

Differentiation from 3,4-dimethoxyphenylethylamine, hydroxymethoxyphenylethylamines and tryptamine This chromatographic pink spot was not produced by d.m.p.e. as the two substances were

separated by chromatography when d.m.p.e. was added to a urinary extract prior to chromatography. An extract similar to that which yielded a pink spot failed to give the specific d.m.p.e. fluorescence by the method of Bell and Somerville (1966b). The method will detect 30µg d.m.p.e./24hr. urine volume as a pink spot on a chromatogram and 12µg (1.5µg in one-eighth of a 24hr. urine collection) as a fluorophor on a chromatogram.

Other pink spot producing compounds with similar R_F values include 4-hydroxy-3-methoxyphenylethylamine and 3-hydroxy-4-methoxyphenylethylamine. Parallel urinary chromatograms treated by the method of Bell and Somerville (1966b) showed a faint yellow fluorescence at the R_F location of the pink spot substance. The characteristics of the fluorescent material eluted from the chromatogram were similar to those for 4-hydroxy-3-methoxyphenylethylamine but its amount was less than 1µg, a quantity insufficient to account for the pink spot reaction. Material with the fluorescence characteristics of 3-hydroxy-4-methoxyphenylethylamine in the Bell and Somerville (1966b) test was apparently absent.

Tryptamine could also be separated from the urinary pink spot, running ahead of it, and therefore could not contribute to the colour of the latter.

Chromatographic and electrophoretic similarity to monoacetyl cadaverine Monoacetyl cadaverine was isographic with the pink spot in n-butanol-acetic acid - water (4:1:1 by vol) (R_F 0.55), isopropanol-ammonia sp.gr. 0.88-water (8:1:1 by vol) (R_F 0.69-0.79) and methanol-pyridine-conc. hydrochloric acid - water (80:10:2.5:17.5 by vol) (R_F 0.91-0.97).

The substance producing the pink spot behaved in the same way during high voltage electrophoresis (HVE) as did that reported by Bell

and Somerville (1966a). Of the substances tested, i.e. 4-methoxyphenylethylamine, 3-hydroxy-4-methoxyphenylethylamine, 4-hydroxy-3-methoxyphenylethylamine, 3,4-dimethoxyphenylethylamine, p-tyramine and monoacetyl cadaverine, only this pink spot producing compound, p-tyramine and monoacetyl cadaverine ran to the same position and just ahead of d.m.p.e., the other compounds migrating to positions behind d.m.p.e. Somerville (personal communication) examined the following and found none of them to migrate as far as d.m.p.e. in the same HVE system (0.2M phosphate buffer pH6.2) :- adrenaline, noradrenaline, dopamine, metadrenaline, normetadrenaline, hydroxymethoxyphenylethylamines, kynurenine pathway metabolites, tryptamine, 5-hydroxytryptamine, melatonin and various other amines.

Effect of gut sterilisation In the case of the one patient examined this pink spot was present during the three consecutive weeks before drugs were administered. Following aerobic and anaerobic culture stool was reported as containing coliforms sensitive to a combination of ampicillin and framycetin. Ampicillin 250mg. 5/day was given for six days and concurrently framycetin 1g. 5/day for five days. A forced diuresis was effected during the last day of drug administration and a 24hr. urine sample collected. A pink spot was still detectable.

Unfortunately the patient would not provide a further stool or allow an enema, so that further culture to prove the efficacy of treatment was not possible. Although the commensal organisms were probably eradicated, this could not be shown definitely and thus the conclusion that pink spot material was not bacterial in origin could not be drawn.

ii. Other pink spots

An orange pink (peach) spot (R_P0.69-0.71) was detected on the chroma-

tograms of two female schizophrenics. The preceding ninhydrin colour was a striking blue. Marker compounds giving this colour sequence and having similar R_F values in n-butanol-acetic acid - water (4:1:1 by vol.) were β-phenylethylamine (R_F 0.73-0.77) and 3-methoxyphenylethylamine (R_F 0.74-0.76). While giving similar colours monopropionyl cadaverine, applied in pure solution or mixed with a parallel urinary extract, did not migrate quite as far as the "peach spot" substance. Although this substance was obtained from each of the six urine samples from those two patients, it was unfortunately not excreted in detectable amounts at a later date so that further characterisation was not possible. It is probable that this material was that referred to as one of the second group of pink spots on chromatograms in the screening study.

In addition to the pink and peach spots just referred to, there were two other spots - a pink and a peach with R_F values of 0.44 and 0.55 respectively - on the chromatograms of one female schizophrenic patient. Although the latter R_F falls within the range given for the principal pink spot, the two substances were separated on this patient's chromatograms.

b) Ion-exchange method Three "pink spot" yielding substances were observed on chromatograms of eluates from the ion-exchange columns developed in n-butanol-acetic acid - water (4:1:1 by vol.).

Relation to urine volume and pH In contrast to the situation obtaining for the solvent extraction procedure, the excretion of substances of interest detected by this method was not related to urine volume or pH.

Compound excreted by all subjects The substance reported in

Sub-section III to have an R_F similar to p-tyramine was detected in this study and was isographic with that compound in n-butanol-acetic acid - water (4:1:1 by vol.) R_F 0.60-0.66, isopropanol-ammonia sp.gr. 0.88-water (8:1:1 by vol.) R_F 0.79, methanol-pyridine-conc.hydrochloric acid - water (80:10:2.5:17.5 by vol.) R_F 0.93, chloroform-methanol-acetic acid - water (65:30:2:3 by vol.) R_F 0.53, and on high voltage electrophoresis in 0.2M phosphate buffer pH 6.2. Paper chromatograms of parallel column eluates developed in n-butanol-acetic acid - water (4:1:1 by vol.) submitted for estimation of p-tyramine by the fluorimetric method always revealed p-tyramine at the R_F location of the pink spot material. However the quantities of p-tyramine detected by fluorimetry in chloroform extracted eluates would not have been sufficient to yield a pink spot of the same intensity as that derived from a parallel extract. This anomaly was resolved when, using the system of Vogel and Ahlberg (1967) i.e. secondary butanol-formic acid 100%-water (40:1:6 by vol.) two pink spots on the chromatograms with R_F values of 0.49 and 0.35 were obtained. The former had an R_F value similar to p-tyramine. The latter substance has not been identified. It was not monoacetyl or monopropionyl cadaverine, m-tyramine, β-phenylethylamine or any of the methoxyphenylethylamines already referred to.

Compounds not excreted by all subjects One giving a reddish-pink chromatographic spot had an R_F of 0.68-0.71 and was present in the urine of two schizophrenics and one normal child aged 6, whose urine was studied incidentally. Another, of the same hue, with R_F 0.58-0.59 was detected in the urine of one schizophrenic and one control. These R_F values refer to chromatograms developed in the n-butanol-acetic acid - water (4:1:1 by vol.) system and the spots were clearly distinguishable from the main pink spot.

Failure to detect 3,4-dimethoxyphenylethylamine Using the method described by Bell and Somerville no d.m.p.e. was detected in the eluates derived from many of the urines. 1-2µg of d.m.p.e. added to the volume of urine (100ml.) routinely extracted could be detected by this method.

Failure to detect an acetyl cadaverine-like compound No material similar to the principal "pink spot" compound obtained by solvent extraction was detected by this method. If this latter substance was monoacetyl cadaverine the finding is explicable since as noted in Sub-section III this amine was found to be eluted from the strong cation exchange resin by sodium acetate which was applied to the resin before the final eluting agent, ammoniacal ethanol.

DISCUSSION

The subjects in this study were schizophrenic and non-schizophrenic patients and healthy nurses. In no circumstances was the detection of a pink spot material found to be related to the diagnostic category. The principal chromatographic "pink spot" obtained by a solvent extraction method was not 3,4-dimethoxyphenylethylamine or p-tyramine, nor did tryptamine contribute to its colour. This "pink spot" material and monoacetyl cadaverine migrated in an identical manner in several paper chromatographic solvent systems and on high voltage electrophoresis and neither substance appeared in the final eluate from a strong cation-exchange resin. Increase in the fluid intake and consequent urinary output of apparent non-excretors of this "pink spot" material was associated with the appearance of the material on the chromatogram.

The principal "pink spot" producing compounds in eluates from a strong

cation-exchange resin were p-tyramine and an unidentified substance which was isographic with p-tyramine in all but one of the paper chromatographic solvent systems employed. These substances were detected in the urine of all subjects irrespective of fluid intake.

The investigations demonstrated that "pink spot" producing compounds are obtainable from urine by solvent-extraction and ion-exchange chromatographic techniques but that those peculiar to one method are not necessarily identical with those detected by the other. This is exemplified by the detection of p-tyramine in eluates from a cation-exchange resin and failure to detect it in urinary extracts obtained by a solvent-extraction procedure. The finding that the pink spot materials obtained by the two different methods were not the same is in contrast to the conclusion of Pind and Faurbye (1966) that the compounds obtained by Friedhoff and Van Winkle (1962) and by Takesada et al (1963) were identical.

The methods employed by previous workers for separation of "pink spot" producing compounds and other substances present in the organic solvent extract or exchange resin eluate have been paper chromatography, thin layer chromatography, high voltage electrophoresis and gas liquid chromatography. Although thin layer chromatography (TLC) has been claimed to be superior to paper chromatography for achieving optimum separation, it is interesting that the authors (Kuehl et al 1964) of the TLC method found a significant number of schizophrenic patients to excrete a pink spot producing compound, thought to be d.m.p.e., but were unable to detect any of this compound in a later study (Kuehl et al, 1966). The criticism of poor separation achieved by paper chromatography would not appear to be applicable to this study. The long chromatographic runs allowed separation of the principal

pink spot material obtained by the solvent procedure from compounds with not very dissimilar R_F values i.e. p-tyramine, d.m.p.e. and tryptamine. The advantage of high voltage electrophoresis over paper chromatography is the separation of phenothiazine metabolites from other pink spot producers. As the patients had received no phenothiazines for at least one year before the initial survey or thereafter until completion of the study, this method offered no advantage as a routine procedure. As there were no reliable clues as to the identity of the pink spots detected in the screening study, the use of gas liquid chromatography was not feasible.

In the screening study, using the solvent extraction method, two groups of pink spots with markedly different paper chromatographic characteristics were found but the incidence of each in schizophrenic and non-schizophrenic psychiatric patients was essentially similar. That with the lower rate of migration was in all probability the same as the principal pink spot detected in extracts obtained by the solvent procedure from the urines of the subjects in the final study.

In this more detailed study of eight patients and three healthy nurses, solvent extraction yielded several pink spots. Association of detection of the most frequently observed "pink spot" material (R_F 0.51 - 0.57 in n-butanol-acetic acid - water (4:1:1 by vol.)) and urine volume has been demonstrated in that its excretion during forced diuresis was detected in all subjects who were apparently non-excretors on a restricted fluid regimen. Although affected by urine volume the excretion of this substance must have varied with other unidentified influences. That this was so was concluded from its intermittent detectability in the urine of the first two subjects in the screening study and the fact that persons who had not excreted detect-

able amounts during the screening survey did so in the more detailed study on a restricted fluid regimen and vice-versa. The conversion from "negative excretor" to "positive excretor" implied a higher incidence of detectable excretion by patients than was indicated by examination of the urine of each on only one occasion during the screening study. Evidence demonstrating the similarity of behaviour of this substance and monoacetyl cadaverine in three solvent systems and on high voltage electrophoresis has been presented. Of course, co-chromatographic studies do not establish the identity of a substance and no claim as to identification is being made. As pointed out by Perry et al (1967) monoacetyl cadaverine is poorly recovered by the solvent extraction method, the figure as estimated in this investigation being in the region of 1%. Nevertheless it is possible that a sufficient quantity of this compound is excreted to allow of its detection. However, there does not appear to be any published data on this subject. This pink spot, whatever its nature, is very likely to be the same as that detected by Bell and Somerville (1966a) and Bourdillon et al (1965). This conclusion is based on the identical behaviour during high voltage electrophoresis of the pink spot material detected in this study and that of Bell and Somerville and the fact that "pink spot positive" urines were supplied to Bell and Somerville by Bourdillon's group. It is difficult to relate the pink spot compound to that of other investigators since many fail to quote R_F values. Since Sen and McGeer (1964) give the R_F of d.m.p.e. in n-butanol-acetic acid - water (4:1:1 by vol.) as 0.58 and Creveling and Daly (1967) also as 0.58 (as reported earlier in the text the R_F values for d.m.p.e. in pure solution in this solvent system was 0.66 for the front of the spot and 0.63 for the centre) it is quite possible that the substance producing

the "pink spot" detected in this study was the same as that found by other investigators, including Friedhoff and Van Winkle.

As d.m.p.e. is a derivative of dopamine and haloperidol interferes with dopamine metabolism, it might be argued that the subjects receiving this medication were unable to synthesise d.m.p.e. Bruno and Allegranza (1965) demonstrated that the effect of administering large doses of the drug to schizophrenic subjects was an increase in urinary levels of homovanillic acid, a derivative of dopamine, during treatment and an augmentation of the urinary content of dopamine for several days after cessation of treatment. In this study two of the eight subjects had received the drug but therapy was stopped two weeks before admission to the ward. These were the two schizophrenics in whose urine the principal pink spot substance was detected during fluid restriction. It seems unlikely that the previous administration of haloperidol influenced the findings of the study in any way.

An orange pink (peach) spot was detected on the chromatograms of two schizophrenics. Compounds with similar R_F values and giving a similar colour sequence with the localisation reagents were β-phenylethylamine, 3-methoxyphenylethylamine and monopropionyl cadaverine. The compound 4-methoxyphenylethylamine tentatively identified in urine by Sen and McGeer (1964) gave a reddish-pink and could not be confused with the "peach spot" substance. β-phenylethylamine has been detected in normal urine by Jepson et al (1960). The urinary excretion of compounds as their propionyl derivatives does not seem to be a normal biochemical mechanism. Moreover although the R_F values were approximately equal the "peach spot" substance migrated slightly further than did monopropionyl cadaverine applied either in pure solution or mixed with a parallel urinary

extract. Further identification was unfortunately not possible as both subjects failed to excrete this substance in detectable amounts at a later date.

In those eight patients and three nurses ion-exchange chromatography yielded pink spot material with the R_F value of and giving the fluorescent reaction for p-tyramine in all subjects. However, although behaving as one substance in four solvent systems, this spot was resolved into two in another system, one of the spots being produced by p-tyramine and the other by an unidentified substance. The finding, employing fluorimetric assay, that only about 10% of p-tyramine eluted from the ion-exchange column is extracted into chloroform after dissolving the dried eluate in pH 9.0 buffer probably accounts for the failure of Takesada et al (1963) to find their pink spot reactor, which was probably compounded of p-tyramine and this other substance, in the urine of 100% of their subjects. The reason for this latter is that p-tyramine excretion varies considerably from subject to subject as will be demonstrated in Section B. Von Studnitz and Nyman (1965), using the ion-exchange method of Takesada et al, found in the urine of schizophrenic patients and normal control subjects pink spot producing material which was no longer excreted on a glucose diet. Since this latter is a diet containing no protein and De Quattro and Sjoerdsma (1967) have demonstrated that tyramine excretion is reduced under such conditions, it seems probable that the pink spot of Von Studnitz and Nyman was also derived from p-tyramine, possibly in conjunction with this other substance. Boulton et al (1967) chose a schizophrenic and four patients with Parkinsonism who had pink spots on their chromatograms of urinary extracts obtained by the solvent extraction method of Friedhoff

and Van Winkle and proceeded to extract pink spot producing compounds from their urine by ion-exchange chromatography, demonstrating by mass spectrometry and other techniques that the substance was p-tyramine. The inference was that pink spot material extracted by the solvent method was also p-tyramine. It is considered that this study demonstrated fairly convincingly that this could not have been so.

Failure to detect, by this method, the main "pink spot" compound obtained by solvent extraction may be explained if this were an aliphatic base such as monoacetyl cadaverine since, as shown by Kakimoto and Armstrong (1962) and confirmed in this study for monoacetyl cadaverine, such substances leave the ion-exchange resin in the sodium acetate effluent before elution with ammoniacal ethanol. However these compounds would have been present in the eluates obtained by Perry et al (1967) who used 4N acetic acid to elute bases from a weak cation exchanger.

The ephemeral nature of the occurrence of the other "pink spot" reacting compounds, occasionally noted in ion-exchange eluates and solvent extracts from urine of subjects and unrelated to clinical status since they were present in the urines from schizophrenic and non-schizophrenic controls, has not allowed their further characterisation.

In this study there has been found to be no significant difference in incidence of excretion by schizophrenic and non-schizophrenic subjects of substances giving the pink spot reaction and in particular of that one which may be identical with the compound of Friedhoff and Van Winkle (1962), Bourdillon et al (1965) and various other investigators. The presence of pink spots on the urinary chromatograms from nurses who only lived in the ward during the 24hrs. of collection suggests that excretion of the pink spot

reacting substance or a precursor of it is not related to long term hospitalisation.

It is unlikely to be of significance that only chronic schizophrenic subjects were investigated because Bourdillon et al found their pink spot on chromatograms of both acute and chronic schizophrenics, and several workers including Pind and Faurbye (1966) examined the urine of chronic schizophrenics by the method of Friedhoff and Van Winkle and found pink spots with the R_F of d.m.p.e. on their chromatograms.

This study, like several others, has yielded no support for the hypothesis that d.m.p.e. is excreted only by schizophrenic subjects and that it is in some way related to their psychoses. However, Creveling and Daly (1967) claim to have identified d.m.p.e. by mass spectrometry in pooled urine from schizophrenic subjects. Although this was present only in small amounts it is possible that there would be sufficient to affect brain metabolism. That orally administered d.m.p.e. does not have this effect could be due to its failure to cross the blood-brain barrier. While no d.m.p.e. was detected in urine in this study Rinne and Sonninen (1967) report finding it in the urine of patients with Parkinsonism and other neurological diseases, employing Bell and Somerville's technique (1966a). However in a recent investigation (Rinne 1969 personal communication) in which they found pink spots on paper chromatograms from the urine of eleven of twenty seven patients with Parkinson's disease and five of twelve control subjects they could not demonstrate the presence of d.m.p.e. in those pink zones when the latter were eluted and re-run on thin-layer silica gel plates. Although the possibility remains that only schizophrenic subjects form d.m.p.e. within their central nervous systems and

excrete small amounts in the urine, the bulk of the evidence to date suggests that this is not so.

A short summary of the findings related to excretion of pink spot material was published in a Letter to the Editor, Nature, Vol. 221, page 971, March 8, 1969.

SECTION B

URINARY EXCRETION OF SOME AMINES AND ACIDS OF PHYSIOLOGICAL IMPORTANCE BY SCHIZOPHRENIC AND HEALTHY SUBJECTS

INTRODUCTION

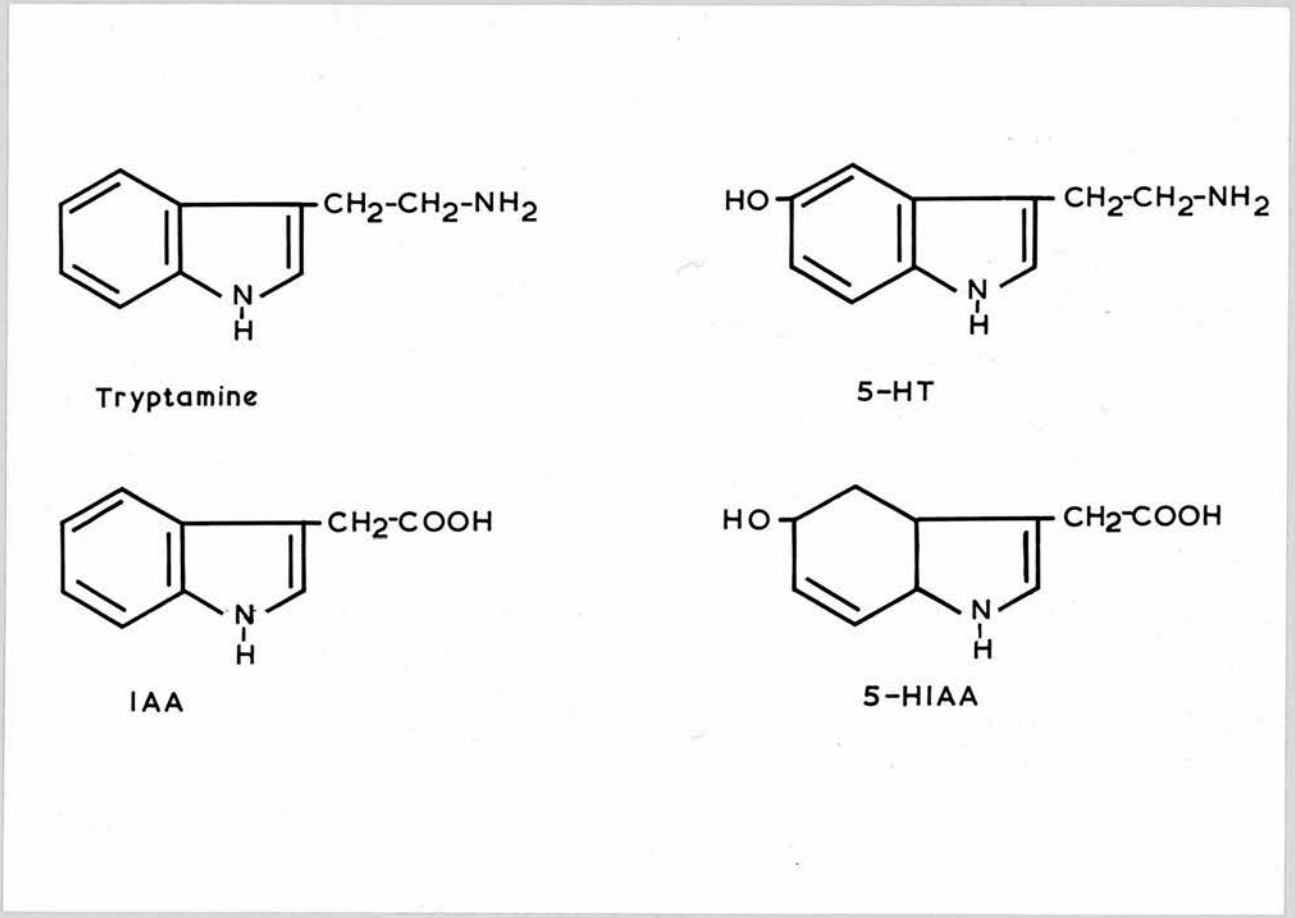
While the work recorded in Section A was concerned with compounds related in structure to adrenaline and noradrenaline the aspect of the study discussed here largely entailed consideration of substances with the indole configuration (fig. 5). There is a considerable body of evidence, too extensive to tabulate, to support the hypothesis that 5-hydroxytryptamine (5-HT) is a chemical transmitter of nerve impulses within the central nervous system. Dewhurst (1968) recently suggested that tryptamine is also a neurotransmitter. However Eccleston et al (1966) demonstrated that evidence for the presence of tryptamine in brain was based on a methodological error. They were unable to detect the amine in animal brain and more recently (personal communication) in human brain.

As for the β-phenylethylamine group, so some indole derivatives have hallucinogenic properties and it has been suggested by Brune and Himwich and co-workers (e.g. Brune et al 1963) that substances such as bufotenin may be responsible for schizophrenic psychosis.

Various groups of workers including Brune and Himwich (1962) have reported a significant positive correlation between urinary excretion by schizophrenic subjects of the indoles, tryptamine and its metabolic end-product, indol-3-yl-acetic acid (IAA), and creatinine and suggested that these substances all find their origin in increased protein catabolism.

Fig. 5

Chemical structures of tryptamine, 5-HT, IAA and 5-HIAA



Abbreviations used:-

5-HT:- 5-hydroxytryptamine

IAA:- indol-3-yl-acetic acid

5-HIAA:- 5-hydroxyindol-3-yl-acetic acid

The same group and others have also noted an increase in the urinary excretion of those indoles during psychotic episodes occurring in schizophrenic patients, although there is some doubt as to whether those episodes were due to the schizophrenic illness or were toxic confusional reactions to drugs such as methionine administered in the course of the study.

As already recounted in Section A Boulton et al (1967) reported that a schizophrenic subject and four patients with Parkinsons disease excreted unusually large quantities of p-tyramine in their urine and suggested that this finding was related to the detection of pink spot material in urinary extracts obtained from these subjects.

The urinary excretion of some weak acids and bases is known to be related to urinary pH (Milne et al 1960). There appear to be two principal mechanisms involved in the renal handling of such substances (Weiner and Mudge 1964). Active proximal tubular secretion is not affected by urinary pH whereas passive non-ionic diffusion in more distal tubular segments is subject to such control. pH dependence is most firmly established for compounds of pharmacological interest but has also been demonstrated in the case of several metabolites of physiological significance. Thus the variable excretion of IAA on this basis has been proven (Milne et al 1960). However there is no similar data available for tryptamine or p-tyramine. The greater the numerical difference between the pK_a value of a weak acid or base and the pH value of blood the more likely is its excretion to be urine pH dependent, providing that other factors such as lipid solubility are favourable. One might thus expect that the excretion of tryptamine, which has a pK_a of 10.2, would be influenced by the degree of urinary

acidity or alkalinity.

The excretion of urinary solutes may also be subject to variation in consequence of changing fluid output. The volume of renal tubular fluid dictates the concentration of the solute and the concentration gradient from within the tubule to the peritubular fluid determines the degree and rate of reabsorption. Urine volume dependence has been demonstrated for noradrenaline by Dawson and Bone (1963) and for chlorpheniramine by Beckett and Wilkinson (1965). Dewhurst (1965) found the excretion of IAA to be related to urine volume rather than to the pH of that fluid.

Purpose of the study

In view of the report of Boulton et al (1967) it was considered important to determine independently whether or not urinary excretion of p-tyramine by schizophrenic patients differed significantly from that of control subjects.

Although the excretion of tryptamine and IAA has been correlated with psychotic episodes in schizophrenia the influence of another variable factor, urinary pH, might alter the interpretation of an apparently simple relationship. Thus an investigation was devised to examine the effect of urinary pH on tryptamine excretion.

Since the relationship of urine volume and the detectability of pink spot substances was being studied in parallel, the variation of tryptamine and p-tyramine with the quantity of urine excreted was also examined.

For purposes of comparison 5-hydroxytryptamine (5-HT) excretion was estimated. The egress of this substance from the body is known not to be affected by urinary pH (Weiner and Mudge 1964).

In order to obtain a dynamic picture of the metabolism of these amines it was proposed to estimate their acid metabolites, IAA from tryptamine, 5-hydroxyindoleacetic acid (5-HIAA) from 5-HT and p-hydroxyphenylacetic acid from p-tyramine. However there was no satisfactory method available for measurement of p-hydroxyphenylacetic acid so that an attempt to devise one was necessary.

Methods

Subjects

The subjects were the four schizophrenic and four non-schizophrenic patients mentioned in Section A, Sub-section IV. Four nurses were studied as controls, three of them being those referred to also in Section A, Sub-section IV. The dietary, fluid and drug regimens have already been described. Some of the foodstuffs excluded from the diet contained the substances to be estimated or precursors of those substances. The regimens were designed to produce acid or alkaline urine and small or large urine volumes.

Analytical procedures

Aliquots of the 24 hr. urine samples were filtered, adjusted to pH 3-4 (glass electrode) with conc. hydrochloric acid and stored at -20°C until examined for amine and acid content within one week.

a) Amines

Free and total p-tyramine, tryptamine and 5-HT were estimated. Conjugated amines were released by the use, applied to different samples, of both enzymatic and acid hydrolysis. Enzymatic hydrolysis was effected by the incubation of 10ml. urine buffered to pH 4.7 with 0.5M acetate buffer with 1,000 units of β-glucuronidase for 18hr. at 37°C. The enzyme preparation used also possessed some sulphatase activity. Acid hydrolysis

was achieved by adjusting 10ml. urine to pH 1.0 (glass electrode) with conc. sulphuric acid and heating in boiling water for 30min. in a sealed glass ampoule containing an atmosphere of nitrogen.

Free and conjugated amines were determined as enumerated in the paper of Le Gassicke et al (1965). Amberlite CG 50 resin, 100-200 mesh, prepared in the ammonium form and buffered to pH 7.5 with 0.2M ammonium acetate buffer was used after settling to a height of 7cm. in glass columns with an internal diameter of 0.6cm. Prior to use the resin was washed with 15ml. 0.02M ammonium acetate buffer pH 7.5. Urine, stored at -20°C, was allowed to thaw at 4°C for 24hr. and adjusted to pH 7.5 (glass electrode). 10ml. samples of urine followed by 15ml. 0.02M ammonium acetate buffer pH 7.5 and 4ml. 0.1N sulphuric acid were allowed to pass through the column at a rate of 10ml./hr. The amines were eluted with 6ml. 1N sulphuric acid at a flow rate of 5ml./hr. (Preliminary experiments indicated that more rapid flow rates gave inconsistent results). The eluates were shaken to ensure mixing and divided into portions, 1ml. for 5-HT, 2ml. for p-tyramine and 3ml. for tryptamine estimation.

5-HT estimation To the 1ml. portion of the eluate was added 0.5ml. conc. hydrochloric acid and the fluorescence measured at 300mμ excitation/535mμ fluorescence.

Para-tyramine estimation The p-tyramine content of the 2ml. portion of eluate was determined as described in Section A, Sub-section III.

Tryptamine estimation The 3ml. portion of the eluate was mixed with 1ml. 5N sodium hydroxide and the tryptamine extracted into 20ml. benzene by shaking for 10min. The amine was then transferred from the benzene to 2.5ml. 0.1N sulphuric acid by shaking for 10min. To 2ml. of the sulphuric

acid after shaking was added 0.1ml. 12% formaldehyde. After heating in a boiling water bath for 20min. and cooling, 0.1ml. 11vols. % hydrogen peroxide was added and the solution again heated in the bath for 20min. Following cooling the norharman fluorophor formed was measured at 360mμ excitation/ 440mμ fluorescence.

N.B. Once the techniques for hydrolysis, development of the columns and the determinations had been deemed satisfactory a technician employed by the M.R.C. Unit for Research in Brain Metabolism carried out the procedures with the exception of hydrolysis.

b) Acids:- IAA and 5-HIAA

Free and total IAA and 5-HIAA were measured. Only acid hydrolysis was employed. For the estimation of the free acids 7ml. urine were used and for the measurement of total acids only 5ml. Acid hydrolysis was effected by the same means as for the amines but the pH was less than 1.0, 0.15ml. conc. sulphuric acid being added.

IAA was estimated by the method of Weissbach et al (1959) and 5-HIAA by the procedure of Udenfriend et al (1955) with the omission of dinitrophenylhydrazine treatment. The sample was adjusted to pH 4.0 and shaken successively with 20ml. and 10ml. chloroform. The chloroform was shaken with 2ml. 0.1M potassium phthalate buffer pH 4.0 and then with 3ml. 0.5M phosphate buffer pH 7.9. To 2.4ml. of the phosphate buffer was added 2.4ml. conc. hydrochloric acid and 6ml. 0.1% xanthydrol in glacial acetic acid. After 10min. 3ml. 5% sodium metabisulphite were added and after a further 5min. the IAA content was determined in a Unicam S.P. 600 spectrophotometer by measuring the optical density at 525mμ.

The urine from which IAA had been removed by chloroform extraction was saturated with sodium chloride and acidified by the addition of 0.5ml. conc. hydrochloric acid. Diethyl ether was shaken with a solution of ferrous sulphate in order to reduce any peroxides present and distilled. The urine was shaken successively with 20ml. and 10ml. of this freshly-distilled ether and the organic solvent then shaken with 2.5ml. 0.5M phosphate buffer pH 6.5. 2ml. of the buffer were removed and to it added 1ml. 0.1% α-nitroso-β-naphthol in ethanol and 1ml. of nitric acid reagent. This latter was freshly prepared by mixing 10ml. 2N sulphuric acid and 0.4ml. 2.5% sodium nitrite. The sample was then heated in a water bath at 55°C for 10 min. following which the excess nitrosonaphthol was extracted into 5ml. dichloroethane. The 5-HIAA content of the aqueous phase was determined by reading the optical density at 540mμ.

c) Attempt to devise a method for the estimation of p-hydroxyphenylacetic acid

This investigation was limited to an attempt to develop a method which was not time-consuming. This stricture was unfortunately necessary as my time was almost fully occupied by the other procedures undertaken. The technique would be required to separate the acid from neutral and basic substances. In addition, depending upon the mode of detection and measurement, it might be necessary to separate it from other phenolic acids. The procedure for measurement would, of course, require to be reproducible and accurate.

Two methods of separation were considered. The first was that of Armstrong et al (1956). The essence of this was successive extraction from urine at pH 1.5 into ethyl acetate, from ethyl acetate into sodium bicarbonate solution and from the alkaline solution, suitably acidified,

Method for extraction from urine and quantitative estimation of p-hydroxyphenylacetic acid

Adjust 7ml. filtered urine to pH 4.0 with hydrochloric acid

↓

Extract with 20ml and then with 10ml chloroform → discard chloroform

↓

Saturate urine with sodium chloride

↓

add 0.5ml conc. hydrochloric acid

↓

extract with 20ml and then with 10ml freshly-distilled diethyl ether

↓

Pool ether and reduce to dryness under a jet of nitrogen

↓

dissolve in 0.05ml methanol

↓

apply to Whatman No. 1 chromatography paper, drying with nitrogen and also apply in parallel known amounts of p-hydroxyphenylacetic acid

↓

Dip paper through α-nitroso-β-naphthol-nitric acid reagent

↓

allow excess reagent to drain for a few minutes

↓

heat paper in chromatography oven at 130°C for 15 minutes

↓

view under ultra-violet lamp

↓

Mark and cut out fluorescent areas

↓

elute fluorophor with 2ml distilled water at 4°C for 1 hour (organic solvents and aqueous/organic solvents and longer elution times produced no improvement in degree of elution)

↓

Read in a fluorimeter at 465mμ excitation/565 mμ fluorescence

back into ethyl acetate followed by two-dimensional paper chromatography. Needless to say this was a time-consuming procedure but it was hoped to shorten it.

The second method consisted of extracting the p-hydroxyphenylacetic acid from urine with IAA or 5-HIAA so that, since these substances were to be estimated in any case, a saving in time would ensue.

Several means of detection and estimation were proposed. However the use of such reagents as brentamine fast scarlet and Folin - Ciocalteau reagent was not feasible as they reacted with the isomers of p-hydroxyphenylacetic acid and also with other phenolic acids. The most likely procedure appeared to be the production of a nitrosonaphthol fluorophor as for p-tyramine. This method has been discussed in detail in Section A, Sub-section III. Following reaction with the reagents the excess nitrosonaphthol was removed by extraction into dichloroethane. Unfortunately as the aqueous phase was acidic this procedure would also remove the fluorescent product derived from p-hydroxyphenylacetic acid (Udenfriend 1962). In order to recover it back-extraction into an alkaline solution would be required.

The separation method of Armstrong et al (1956) was examined. However it was found that a considerable number of other substances were in close proximity on the chromatogram so that choice of the correct area of paper for elution would have been difficult. In order to overcome this problem the fluorescence reaction was developed on the paper as described in Section A, Sub-section III. Although various aqueous and organic solvents were used it was not feasible to elute the fluorophor as only a small and variable proportion could be extracted from the paper. As for p-tyramine Boulton employed one-dimensional paper chromatograhpy and development of the fluorophor on the paper with subse-

quent determination by his strip scanning device (personal communication). Unfortunately this option was not open to me.

Employing the extraction procedure used for IAA and 5-HIAA it was found that p-hydroxyphenylacetic acid passed into ether. In order to avoid immediate consideration of a means of recovering the nitrosonaphthol fluorophor from the dichloroethane it was decided initially to reduce the ether extract to dryness under nitrogen, dissolve the residue in methanol, apply the extract to paper, develop the fluorophor on paper and then elute it, despite the poor and variable recovery at this final stage. In the event, it was found that sufficient of the fluorophor could be eluted for evaluation of the method. The assessment was that the procedure was unsatisfactory because interfering material caused quenching of the fluorescence and also gave fluorescence at wavelengths close to that of the p-hydroxyphenylacetic acid fluorophor emission.

As a satisfactory procedure had not been devised it was unfortunately not possible to proceed with the original intention of studying the acid derivative of each of the three amines.

RESULTS

Amines

The results of this aspect of the study were disappointing. The execution of the procedures rather than the conclusions drawn from the investigation was the disturbing feature. As has already been stated the ion-exchange column development and the fluorimetric assays were carried out by a technician. Those procedures have given satisfactory results in other hands so that one can only conclude unhappily that the fault did not lie with the materials or the methods used.

Many investigators do not routinely add to samples, before processing, a known amount of the substance to be determined in order to gauge the degree of recovery and thus correct the results according to this factor. However in this investigation every set of samples included unhydrolysed, enzyme hydrolysed and acid hydrolysed aliquots of urine to which had been added predetermined quantities of each amine. It was the considerable variation in recovery from week to week and the low recoveries which indicated that all was not well. The recoveries of p-tyramine from unhydrolysed, enzyme hydrolysed and acid hydrolysed urine were 42 ± 21%, 40 ± 22% and 26 ± 13%, of tryptamine 38 ± 13%, 34 ± 12% and 26 ± 10%, and of 5-HT 68 ± 22%, 59 ± 16% and 43 ± 16%. These figures refer to mean recoveries ± one S. D. (26 observations).

Nevertheless when large quantitative differences were obtained in the course of the study tentative conclusions could be drawn. All figures have been corrected according to the appropriate mean recovery for the entire series of estimates extending over a thirty six week period. The 24hr. urinary values for each amine for each subject are given in tables 9, 10 and 11. The details of urine volume and pH are included in table 12. It will be noted that no attempt has been made to conceal discrepancies.

The same operator determined the amine content of eight identical samples of unhydrolysed urine and the same number of samples of enzymatically and acid hydrolysed urine. In order that the significance of each estimation in relation to others can be seen the following standard deviations, obtained from the above data, should be noted. Each standard deviation is given as a percentage of the value to which it refers. Unhydrolysed urine :- p-tyramine 26%, tryptamine 14%, 5-HT 17%; enzyme hydrolysed urine :- p-tyramine 12%, tryptamine 26%, 5-HT 19%; acid hydrolysed urine :- p-tyramine 18%, tryptamine 16%, 5-HT 20%. It will be seen that there is not an orderly

TABLE 9 24hr. urinary p-tyramine (µg) Amounts present in untreated and hydrolysed urine

Schizophrenic subjects (A - D)

	A				B				C				D			
	Ac.	Alk.	R	D	Ac.	Alk.	R	D	Ac.	Alk.	R	D	Ac.	Alk.	R	D
Unhydrolysed	590	530	670	790	860	700	1000	730	480	450	590	1200	220	280	370	170
Enzyme	590	660	890	1000	1300	440	900	980	620	460	700	980	290	270	560	600
Acid	1100	1600	1200	1100	1600	1000	1300	1700	830	lost	1500	2350	400	480	550	1500

Control patients (E - H)

	E				F				G				H			
	Ac.	Alk.	R	D	Ac.	Alk.	R	D	Ac.	Alk.	R	D	Ac.	Alk.	R	D
Unhydrolysed	850	350	790	650	600	240	400	710	790	130	380	360	280	250	370	300
Enzyme	750	610	790	890	690	240	500	620	670	120	410	590	280	310	410	380
Acid	1000	800	1500	1000	900	750	1160	800	1400	300	1600	400	600	650	700	600

Control nurses (N - R)

	N				P				Q				R			
	Ac.	Alk.	R	D	Ac.	Alk.	R	D	Ac.	Alk.	R	D	Ac.	Alk.	R	D
Unhydrolysed	250	460	490	300	600	410	670	370	800	650	780	800	170	390	220	540
Enzyme	310	460	630	180	400	290	520	820	920	670	800	1100	200	690	230	900
Acid	700	1400	1100	900	2300	1700	2400	2400	1300	1000	1400	1700	350	1400	320	1700

Abbreviations Ac.- acid urine; R - urine when fluid intake restricted.
Alk.- alkaline urine; D - urine when fluid intake excessive (diuresis)

TABLE 10 24hr. urinary tryptamine (µg) Amounts present in untreated and hydrolysed urine

Schizophrenic subjects (A - D)

	A				B				C				D			
	Ac.	Alk.	R	D	Ac.	Alk.	R	D	Ac.	Alk.	R	D	Ac.	Alk.	R	D
Unhydrolysed	190	5	75	60	45	Nil	5	75	290	45	65	150	35	5	90	50
Enzyme	210	25	110	30	190	detected in	85	5	100	55	120	90	40	80	55	140
Acid	200	40	100	70	180	10 ml.	100	65	190	lost	90	70	55	90	65	100

Control patients (E - H)

	E				F				G				H			
	Ac.	Alk.	R	D	Ac.	Alk.	R	D	Ac.	Alk.	R	D	Ac.	Alk.	R	D
Unhydrolysed	210	25	120	90	110	10	20	30	20	20	140	5	15	25	20	25
Enzyme	260	40	110	130	60	25	55	65	75	30	90	160	15	20	45	50
Acid	120	80	180	110	140	70	40	90	140	120	180	65	30	30	75	60

Control nurses (N - R)

	N				P				Q				R			
	Ac.	Alk.	R	D	Ac.	Alk.	R	D	Ac.	Alk.	R	D	Ac.	Alk.	R	D
Unhydrolysed	80	40	70	130	150	40	130	55	270	5	110	110	270	15	170	70
Enzyme	95	45	85	95	150	55	210	130	230	80	130	150	370	0	220	200
Acid	155	45	85	120	130	100	110	130	570	120	100	240	260	5	190	150

Abbreviations Ac. - acid urine; R - urine when fluid intake restricted.
Alk. - alkaline urine; D - urine when fluid intake excessive (diuresis)

TABLE 11 24hr. urinary 5-HT (µg) Amounts present in untreated and hydrolysed urine

Schizophrenic subjects (A - D)

	A				B				C				D			
	Ac.	Alk.	R	D	Ac.	Alk.	R	D	Ac.	Alk.	R	D	Ac.	Alk.	R	D
Unhydrolysed	70	30	80	80	40	140	70	180	120	100	250	130	60	70	110	60
Enzyme	80	60	90	90	140	140	160	180	130	100	270	210	70	80	100	130
Acid	200	190	200	260	280	310	210	590	190	lost	310	430	170	260	200	250

Control patients (E - H)

	E				F				G				H			
	Ac.	Alk.	R	D	Ac.	Alk.	R	D	Ac.	Alk.	R	D	Ac.	Alk.	R	D
Unhydrolysed	190	110	190	140	310	130	160	80	30	30	140	140	50	90	120	50
Enzyme	210	130	160	180	390	130	170	120	120	200	180	230	80	110	150	70
Acid	400	360	400	430	1220	330	310	390	390	320	550	610	190	290	410	100

Control nurses (N - R)

	N				P				Q				R			
	Ac.	Alk.	R	D	Ac.	Alk.	R	D	Ac.	Alk.	R	D	Ac.	Alk.	R	D
Unhydrolysed	130	70	130	180	110	130	290	150	130	100	160	240	220	280	200	130
Enzyme	160	100	140	170	130	120	260	250	130	120	220	340	240	260	200	200
Acid	520	220	300	440	300	330	1100	530	440	330	380	690	450	660	340	410

Abbreviations Ac. - acid urine; R - urine when fluid intake restricted.
Alk. - alkaline urine; D - urine when fluid intake excessive (diuresis)

TABLE 12 24hr. urine volume and pH produced by the four regimens

Subject	Urine acid		Urine alkaline		Fluid restricted		Fluid excessive	
	Vol.(ml)	pH	Vol.(ml)	pH	Vol.(ml)	pH	Vol.(ml)	pH
A	1085	5.3	1155	8.2	1135	6.0	1970	6.8
B	1450	5.2	865	8.1	865	6.1	1650	6.8
C	1420	5.0	1340	8.3	1020	6.5	1400	6.3
D	820	6.2	1085	7.3	880	6.7	1680	6.7
E	1175	5.3	1070	8.2	985	6.5	1370	6.3
F	1655	5.8	805	8.0	1070	6.9	1735	6.7
G	1340	5.7	1205	7.9	1350	7.3	1960	7.4
H	600	5.6	930	8.4	1045	6.4	1080	6.1
N	1025	5.8	670	8.0	835	6.1	1225	6.0
P	1045	5.3	1095	8.1	1080	6.0	1570	5.8
Q	1430	5.5	1160	8.2	925	6.2	1710	6.4
R	1330	5.2	1675	8.3	1125	5.6	1320	6.7

variation between the recoveries from the urines differently treated.

The tentative conclusions which can be drawn from the data are the following. Acid hydrolysis released conjugated p-tyramine and 5-HT. Referring only to estimations of free tryptamine during the weeks when the urine was at the extremes of acidity or alkalinity there was a distinctly greater excretion in acid than in alkaline urine in the case of nine of the twelve subjects.

Other deductions which required to be tempered with greater caution were that the excretion of amines by schizophrenic subjects did not differ in any way from that by control subjects, that the degree of variation of urine volume encountered in this study did not affect the excretion of any amine markedly and that the excretion of p-tyramine and 5-HT did not vary with urinary pH. In relation to the first observation it will be noted that the greatest output of total p-tyramine was by control Nurse P.

Acids

Estimation of the IAA and 5-HIAA content of eight identical samples of unhydrolysed urine and of another eight hydrolysed samples was carried out in order to determine the standard deviation applicable to each assay. The values obtained were as follows :- IAA in unhydrolysed urine 5%, IAA in hydrolysed urine 7%, 5-HIAA in unhydrolysed urine 13% and 5-HIAA in hydrolysed urine 9%. The larger standard deviation for 5-HIAA in untreated urine compared with that for the acid in the hydrolysed samples was probably due to the fact that the optical densities of 5-HIAA in the former were at the lower limits of instrumental sensitivity and accuracy.

Each batch of samples processed weekly included unhydrolysed and hydrolysed urines containing 30µg added IAA and 5-HIAA. The mean recoveries over

the thirty six weeks and the standard deviations of those values were as follows :- IAA in unhydrolysed urine 72±12%, IAA in hydrolysed urine 72±9%, 5-HIAA in unhydrolysed urine 54±7% and 5-HIAA in hydrolysed urine 34±8%.

The poor recovery of 5-HIAA from hydrolysed urine indicated destruction of the acid and the rather large standard deviation of those recoveries taken in conjunction with the frequent finding of smaller quantities of 5-HIAA in the treated than in the untreated samples from the same subjects seemed to indicate that the degree of destruction was variable. Thus the data referring to total 5-HIAA is unlikely to be reliable.

The 24hr. urinary content of the acids, free, conjugated and total for each subject is presented in tables 13 and 14. The relevant urine volumes and pH have already been given in table 12.

It can be seen from the data that the acid excretion by the schizophrenic patients did not differ from that of the other subjects.

The free IAA dependence on urinary pH was confirmed for all subjects when extremes of pH were considered and for eight of the twelve this relationship held when the pH of all four urine samples were taken into account i.e. the progressive increase in pH values of the four samples was mirrored by a similar rise in IAA content.

In the case of ten of the subjects there was more conjugated IAA in the acid than in the alkaline urine although the finer gradation was not so consistent. This finding suggests a pH dependence for the conjugate in keeping with that of a basic compound i.e. indolylacetyl glutamine. In the acid urines the free IAA comprised 11-29% (mean 20.6%) of the total and 42-70% (mean 54.9%) of the IAA was in the unconjugated form in the alkaline samples.

TABLE 13 24hr. urinary IAA (mg) Amounts present in untreated and hydrolysed urine

Schizophrenic subjects (A - D)

	A				B				C				D			
	Ac.	Alk.	R	D	Ac.	Alk.	R	D	Ac.	Alk.	R	D	Ac.	Alk.	R	D
Free	0.2	12.5	2.7	2.5	4.4	41.0	16.7	29.5	1.6	6.9	3.0	3.0	1.1	4.8	2.7	3.8
Total	13.3	21.3	13.2	12.6	16.4	69.0	40.7	44.5	7.5	9.8	6.9	16.0	3.8	11.4	6.4	8.5
Conjugated	13.1	8.8	10.5	10.1	12.0	28.0	24.0	15.0	5.9	2.9	3.9	13.0	2.7	6.6	3.7	4.7

Control patients (E - H)

	E				F				G				H			
	Ac.	Alk.	R	D	Ac.	Alk.	R	D	Ac.	Alk.	R	D	Ac.	Alk.	R	D
Free	2.0	6.2	5.4	3.1	2.4	4.3	4.7	1.2	10.4	24.0	26.0	23.0	1.0	4.3	2.9	2.4
Total	15.4	10.5	13.8	16.4	9.9	9.3	15.0	6.7	75.0	21.0	65.0	60.0	8.8	9.3	7.9	7.2
Conjugated	13.4	4.3	8.4	13.3	7.5	5.0	10.3	5.5	64.6	-	39.0	37.0	7.8	5.0	5.0	4.8

Control nurses (N - R)

	N				P				Q				R			
	Ac.	Alk.	R	D	Ac.	Alk.	R	D	Ac.	Alk.	R	D	Ac.	Alk.	R	D
Free	3.9	4.7	2.2	2.9	* 0	12.0	4.5	9.5	2.7	4.6	7.5	6.4	1.2	10.0	2.6	3.2
Total	13.3	8.1	6.3	13.8	22.0	22.0	14.8	36.0	14.2	8.6	15.5	13.2	11.0	17.6	12.0	11.0
Conjugated	9.4	3.4	4.1	10.9	22.0	10.0	10.3	26.5	11.5	4.0	8.0	6.8	9.8	7.6	9.4	7.8

Abbreviations Ac. - Acid urine; R - urine when fluid intake restricted.
Alk. - Alkaline urine; D - urine when fluid intake excessive (diuresis)

* None detected in 7ml.

TABLE 14 24hr. urinary 5-HIAA (mg) Amounts present in untreated and hydrolysed urine

Schizophrenic subjects (A - D)

	A				B				C				D			
	Ac.	Alk.	R	D	Ac.	Alk.	R	D	Ac.	Alk.	R	D	Ac.	Alk.	R	D
Free	3.1	3.3	3.5	3.4	3.1	4.3	4.7	3.6	3.2	4.0	4.4	6.9	3.7	4.6	4.2	5.0
Total	5.8	4.9	4.5	5.0	0.4	5.6	6.0	5.0	4.3	3.1	4.3	7.9	4.9	6.8	5.0	6.7
Conjugated	2.7	1.6	1.0	1.6	-	1.3	1.3	1.4	1.1	-	-	1.0	1.2	2.2	0.8	1.7

Control patients (E - H)

	E				F				G				H			
	Ac.	Alk.	R	D	Ac.	Alk.	R	D	Ac.	Alk.	R	D	Ac.	Alk.	R	D
Free	4.0	4.1	4.5	3.7	8.5	2.9	6.2	3.1	4.7	4.0	5.0	4.3	5.1	4.0	4.3	4.4
Total	3.3	1.1	1.9	0.3	9.4	6.6	8.5	7.9	5.1	0.2	7.0	3.4	3.8	5.2	6.2	2.7
Conjugated	-	-	-	-	0.9	3.7	2.3	4.8	0.4	-	2.0	-	-	1.2	1.9	-

Control nurses (N - R)

	N				P				Q				R			
	Ac.	Alk.	R	D	Ac.	Alk.	R	D	Ac.	Alk.	R	D	Ac.	Alk.	R	D
Free	4.1	2.9	4.6	5.7	2.7	4.0	2.3	5.2	4.9	3.0	4.5	4.0	3.6	5.9	4.0	3.7
Total	5.5	2.6	4.6	6.6	1.9	2.6	2.3	10.6	7.2	3.4	5.1	9.4	4.6	4.6	4.5	2.8
Conjugated	1.4	-	0	0.9	-	-	0	5.4	2.3	0.4	0.6	5.4	1.0	-	0.5	-

Abbreviations Ac. - Acid urine; R - Urine when fluid intake restricted.
Alk. - Alkaline urine; D - Urine when fluid intake excessive (diuresis)

There was no consistent variation of either free or total 5-HIAA with urine volume or pH although some fluctuation in excretion, possibly due to inconstant dietary content of precursors, was noted. The unreliable data for total 5-HIAA already referred to would not allow of a decision as to whether there was in fact a conjugate present. The recovery of added 5-HIAA may have differed sufficiently from that of the endogenous acid in the other samples processed at the same time to account for the computation of the apparent levels of conjugate.

Tryptophan load in subject B

The excretion of IAA by subject B, a female schizophrenic patient, was greatly in excess of the normal values quoted by other authors. Among the possible causes were Hartnup disease and malabsorption syndrome. In both of these conditions one would expect to find an abnormal tryptophan load response. Oral DL-tryptophan 20mg./kg. was given after withdrawal of blood when the patient was fasting. Further blood samples were taken after one, two and three hours. Miss A. Urquhart, technician with the M.R.C. unit for Research in Brain Metabolism kindly estimated the levels of plasma tryptophan. The values of tryptophan were 10.0, 57.6, 47.6 and 35.8 µg./ml. plasma at zero, one, two and three hours respectively. These figures were similar to others obtained by Dr. A.T.B. Moir (personal communication) from ten healthy subjects.

Patient B had none of the stigmata of Hartnup disease nor had she suffered from a gastro-intestinal infection.

Patient G, diagnosed as suffering from the effects of frontal lobe injury, also excreted large quantities of IAA but he was not further investigated.

DISCUSSION

A point requiring to be conceded at the outset of a consideration of the results is that the total number of subjects was small and furthermore the number in each group comprised only four. The reason for the failure to study larger samples, which would have given more statistically satisfactory data was the ancillary nature of the investigation, it being complementary to the "pink spot" work. Thus to regard this as a pilot study would be preferable to treating it as a source of indisputable data. Moreover the unsatisfactory quality of the amine determinations, stemming from technician error, rendered any interpretation of those particular figures even more tentative.

The lack of success in developing a short, accurate method for the estimation of p-hydroxyphenylacetic acid was disappointing. It is possible that had more time been available for a determined approach to the problem or had it been feasible to employ a long procedure a technique would have been forthcoming. However the matter was not sufficiently important to warrant further delay in the proceedings. The more time spent in developing methodology meant a greater chance of the subjects of the study becoming unavailable for a variety of reasons.

Boulton et al (1967) reported the detection of "abnormally large amounts of p-tyramine" i.e. 2,211 µg./l.lg. creatinine and 1,289 µg./l.lg. creatinine in the urine of patients with Parkinsons' disease and schizophrenia respectively and correlated this with the excretion of pink spot precursors.

The data in this study is sufficiently reliable to indicate that these amounts were not, in fact, abnormally large. It was noted in Section A that their figures referred to total p-tyramine and it can be seen from

table 9 that nurse P excreted 2,400 µg. of the free and conjugated amines per 24hr. and that the urine of other subjects contained 400-1,700 µg. Asatoor (1968) detected 2.1mg. in the urine of a normal subject. Although in this study some of the apparent p-tyramine in hydrolysed urine may have been p-hydroxyphenylethanolamine and its N-methyl derivative (Udenfriend 1962) this would also have been true of the estimations of Boulton et al. There was no notable difference between the quantities of p-tyramine in the urine of schizophrenic patients and that of control subjects.

No pH dependence was demonstrated for this amine. The favourable pK_a of 10.9 is presumably offset by the presence of a hydroxyl group on the ring decreasing the lipid solubility. It was noted in Section A that the estimation of this pink spot producing compound in the urine of the schizophrenic subjects suggested urine volume dependence of the amine but that the finding may have been an artefact. This surmise proved to be correct as no correlation between p-tyramine excretion and urine volume was demonstrable in this more detailed investigation. It is of course possible that such a relationship could have been masked by the operation of other variables. Thus dietary intake of the amine or of its amino-acid precursors and metabolic activity of intestinal bacteria might affect the urinary output. The origin of urinary p-tyramine is controversial. Awapara et al (1964) and Perry et al (1966) considered that dietary amino-acids and intestinal bacterial flora rather than tissue activity represented the major sources whereas De Quattro and Sjoerdsma (1967) thought that the quantity excreted reflected plasma concentration of the amino-acids and the activities of tissue decarboxylase and monoamine oxidase. Recently Asatoor

(1968) opined that both bacterial and tissue sources might assume significance.

No correlation between the levels of urinary p-tyramine and creatinine was noted.

Sjoerdsma et al (1959) reported 36-120µg tryptamine in the 24hr. urine samples of ten normal subjects but Arterberry and Conley (1967) found a larger range. From the hourly excretion figures quoted by the latter authors daily values were calculated as ranging from 45 to 610µg. Such data however took no account of the urinary pH. Ignoring this factor the comparable figures for free tryptamine in this study were 5-290µg. However in alkaline urine the variation was much less marked i.e. 5-45µg and in acid urine the range was 35-290µg.

The data appears to be sufficiently reliable to support the anticipated finding of dependence of tryptamine excretion on urinary pH. The implication of this relationship is that any future investigation of excretion of this amine should somehow control or allow for urinary pH. Other workers are not unaware of this since Coppen (1967) in a review article reported that he manipulated pH and Faurbye (1968) in a similar paper suggested that such a factor might require to be taken into account. It was noted in the introduction to this Section that various researchers had observed an increased excretion of both tryptamine and IAA by schizophrenic subjects during psychotic episodes and that a rise in urinary creatinine occurred pari passu. No correlation of the excretion of either substance with that of urinary creatinine was detected in this study but it would hardly have been expected since the variation in excretion of the amine and acid resulted from manipulation of their renal handling whereas that of the

acutely psychotic subjects may have originated in some endogenous metabolic change which also affected creatinine formation. That some mechanism other than renal was responsible for the elevation of urinary tryptamine and IAA is apparent since a rise in pH effects a decrease in tryptamine and an increase in IAA output and a fall in pH also affects these substances in a diametrically opposed manner i.e. no alteration in urinary pH can cause a simultaneous rise or fall of the levels of the base and its acid derivative.

In the case of 5-HT Rodnight (1956) detected 1.88-5.0µg/hr. i.e. 45-120µg/24hr. in human urine and Arterberry and Conley (1967) 2.39-9.89µg/hr. which is equivalent to 60-240µg/day. The range for free 5-HT in this study was 30-290µg/24hr. It has already been established (Weiner and Mudge 1964) that the phenomenon of pH dependence does not obtain for 5-HT and this was confirmed in the present study. No evidence of a relationship with urine volume was observed either.

Most of the discussion so far has been confined to a consideration of the free amines. However as one mode of deactivating an amine in the body is the formation of a conjugate one must measure both free and conjugated base in order to determine the total urinary content. That p-tyramine occurs in conjugated form is established. However there is no reference in the literature to conjugated tryptamine or 5-HT. Ashcroft G.W. and Crawford T.B.B. (personal communication) have found no evidence that either substance appears in the urine as a conjugate. Perusal of the data in tables 10 and 11 will reveal that there is no ground for assuming otherwise in the case of tryptamine but that acid hydrolysis released 5-HT from chemical bondage. As the enzyme preparation contained β-glucuronidase and effected no definite release the latter amine is not conjugated as a

glucuronide but as a sulphate or in some other form.

The well-established pH dependence of IAA was confirmed but another observation not referred to in the literature, although it was predictable, was made. This was the variation with pH of the IAA conjugate. Whereas the free acid was excreted in greater amounts in alkaline urine the opposite was the case for the bound form. This was in keeping with the identification of the latter as indolylacetylglutamine by Jepson (1956).

Milne et al (1960) reported that at pH 4.5 free IAA comprised 40% of the total and at pH 8.0 90%. In this study 11-29% (mean 20.6%) of the IAA was in the free form in the acid urine samples and 42-70% (mean 54.9%) in the alkaline. The manner of presentation of the data of Milne et al does not allow of comparison with that obtained in this study for free and conjugated acid. That I obtained smaller proportions of free:total IAA than they did could have been due to one of two factors or a combination of the two i.e. detection of smaller absolute amounts of free IAA or greater absolute amounts of conjugated IAA. As my figures for the free acid were similar to those of Weissbach et al (1959) it is probable that I detected more of the conjugated form. If this was so one possible explanation for the lower values obtained by Milne et al is that they may have destroyed a considerable quantity during acid hydrolysis. As hydrolysis in an atmosphere of nitrogen is not generally practised this could have been the case.

Weissbach et al (1959) obtained values of 3.1-8.1mg/24hr. for the free acid and 5.2-18mg/24hr. for total IAA and Arterberry and Conley (1967) reported total values of 6.15-38mg/24hr. In this study the range of excretion of the free form for ten of the twelve subjects was 0.2-12mg and 3.8-36mg for the free and conjugated forms together. However in the

case of the schizophrenic subject B the amount of free IAA in alkaline urine was 41mg and the total quantity in the same sample 69mg. This patient did not have Hartnup disease, diabetes or any of the other disorders reported by Weissbach et al (1959) to be associated with abnormally high values nor was there any evidence of an intestinal bacterial source as the tryptophan load test gave normal results. Unfortunately this subject was not available for further study. Control Patient G, the subject of frontal lobe injury, excreted 26mg free IAA in one 24hr. sample and 65mg total IAA in another. No further investigations were undertaken to elucidate the findings, mainly owing to lack of free time. That he continued to receive anti-convulsants during the study may have been relevant.

The findings in this study were in agreement with those of Milne et al (1960) who noted that the excretion of 5-HIAA did not vary with urinary pH. No correlation with urinary volume was observed. Udenfriend et al (1955) measured the presence of 2.0-8.2mg 5-HIAA/24hr. in the urine of general hospital patients and Arterberry and Conley (1967) quoted values of 2.0-8.6mg/24hr. for normal subjects. The data obtained from the investigations reported here indicated a range of 2.3-8.5mg. These three sets of figures are in close agreement. The upper limit of excretion of total acid in this study was 10.6mg. However it has already been pointed out in the Results section that the poor recovery of added 5-HIAA from hydrolysed urine, the rather large standard deviation for the recoveries and the frequent finding of smaller quantities of the acid in the hydrolysed urine than in the untreated sample probably indicated variable destruction of the acid under conditions of hydrolysis and that the data was therefore unreliable. Thus there was no convincing evidence that 5-HIAA was present in conjugated

form. This was in keeping with the finding of other investigators. Snow et al (1955) commented that their identification of 5-HIAA sulphate ester in the urine of two patients with metastasising carcinoid tumours was the first record of a naturally occurring 5-HIAA conjugate other than bufothionine which is present in the skin of some amphibia.

FINAL DISCUSSION

Ever since a more humane attitude to psychotic patients was adopted in preference to fettering and bludgeoning it has been realised that such people are afflicted by a particularly disabling and unpleasant disease. Not unnaturally therefore much effort has been expended in pursuit of the elusive aetiological factor and attempts to reach the heart of the matter swiftly are understandable. Unfortunately such haste has been the enemy of progress. It is being increasingly realised that research based on the shifting sands of unproved methods can only lead to disappointment. The clinician who makes light of the requirements of rigorous methodology and the non-medical scientist who is unaware of the snares concealed by all-inclusive diagnostic categories alike will founder. While the "Jack of all trades" in this field has his place he must be able to recognise his shortcomings and work in collaboration with a representative of another discipline.

The first paragraph of this thesis pointed to the necessity of distinguishing between types of schizophrenia. This study has not done so. Ideally acute schizophrenic patients should have been the subjects of the investigation but unfortunately it is exceedingly difficult to marshal more than a few at any one time. The ease with which American psychiatrists seem able to study relatively large numbers of such patients suggests that their diagnostic classification does not entirely coincide with ours. The subdivision of a group of chronic schizophrenic subjects into sets of distinct clinical prototypes is not an easy task. The edges overlap and become blurred in proportion to the duration of the illness. This was the reason for the use of the unqualified terms, "schizophrenia" and

and "schizophrenic", but the deficiencies of the study in this respect are acknowledged.

An attempt has been made not only to avoid the confusion engendered by such influences as uncontrolled diet, medication, urine volume and pH but also to determine the precise effect of some of those factors. Undoubtedly the study has fallen short of the aims so ingenuously defined at the outset but only by the successful application of rigorous methodology in the laboratory and in the ward will much needed advances be achieved.

APPENDIX

Synthesis of Monoacetyl and Monopropionyl Cadaverine Hydrochlorides

Since these substances were not commercially available I had to synthesise them. The procedure was that quoted by Perry et al (1967), a modification of the method of Tabor et al (1964) for the synthesis of monoacetyl putrescine.

5g. cadaverine (1,5-diaminopentane) were added dropwise to 30ml. cooled glacial acetic acid or propionic acid, while stirring continuously. The solution was heated to 55°C and 4.5ml. acetic anhydride or propionic anhydride were added dropwise over the course of 1.5hr. during continuous stirring. The resulting solution was left overnight at room temperature in a glass stoppered flask. The liquid was then reduced to "dryness" in a rotary evaporator at 55°C. The end product was 10-20ml. of a syrupy brown liquid. This was dissolved in 100ml. water and after heating to 55°C 14ml. 6N hydrochloric acid were added slowly during continuous stirring. Evaporation to dryness in a rotary evaporator left a solid brown residue. 200ml. cold isopropanol were added and the mixture refluxed for 20min. The hot isopropanol was vacuum-filtered and reduced in volume to 5-10ml. The precipitate formed after cooling was filtered and redissolved in isopropanol. After five crystallisations from isopropanol a light brown solid was obtained. When dissolved and chromatographed and treated with the pink spot reagents two spots were obtained. In the case of monoacetyl cadaverine the ninhydrin colour of one was purple violet and the Ehrlich salmon pink. The monopropionyl cadaverine gave purple violet followed by orange pink (peach). In both instances the second spot had a low R_F value and did not yield a pink spot. As this substance did not interfere chromatographically, no further purification was

undertaken. Melting points of the hydrochloride derivatives were not determined

REAGENTS

Unless otherwise stated in the text all reagents used were of analytical grade.

Distilled, deionised water was used for the preparation of all aqueous solutions.

Hydrochloric acid:- B.D.H. Micro-analytical reagent.

Ethanol:- Ethanol was refluxed for 2hr. with 20g. potassium hydroxide per 400ml. ethanol and then distilled twice.

Diethyl ether:- Diethyl ether was washed with successive batches of a solution of ferrous sulphate until the latter remained green. The ether was then distilled and used immediately.

Benzene:- Benzene used for tryptamine extraction was washed with 0.1N sulphuric acid.

Drugs:- The methoxylated phenylethylamines were obtained by Dr. J.R. Smythies. They were synthesised as their hydrochlorides by F. Benington, R.D. Morin and L.C. Clark, Jr. of the University of Alabama.

Dopa:- DL-3,4-dihydroxyphenylalanine, Koch-Light Lab.

Dopamine:- 3-hydroxytyramine hydrochloride, Koch-Light Lab.

Tyramine:- Tyramine hydrochloride, B.D.H. Laboratory Chemicals.

m-tyramine:- m-tyramine hydrochloride gifted by Dr. P. Smith, Institute of Aviation Medicine, Farnborough.

Phenylalanine:- L-β-phenylalanine, B.D.H. Laboratory Chemicals.

3-5 diiodotyrosine:- 3,5-diiodo-L-tyrosine, Glaxo Ltd.

Monoacetyl cadaverine:- Monoacetyl cadaverine hydrochloride synthesised as described in the appendix.

Monopropionyl cadaverine:- Monopropionyl cadaverine hydrochloride synthesised

as described in the appendix.

5-hydroxytryptamine:- Serotonin creatinine sulphate, Koch-Light Lab.

Tryptamine:- Tryptamine hydrochloride, B.D.H. Laboratory Chemicals.

IAA:- Indol-3-yl-acetic acid, Koch-Light Lab.

5-HIAA:- 5-hydroxyindolyl-3-acetic acid (diethylammonium salt), Roche Products Ltd.

β-glucuronidase:- β-glucuronidase, Baylove Chemicals Ltd.

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Urine Volume and Pink Spots in Schizophrenia and Health

BECAUSE of conflicting reports about the occurrence of urinary constituents yielding pink colours on chromatograms when treated with ninhydrin followed by Ehrlich's reagent, we investigated these substances excreted by schizophrenic and other psychiatric patients using two extraction procedures in parallel.

Because very few acute schizophrenic patients were available, our subjects were twenty chronic schizophrenic and twenty non-schizophrenic patients. The sexes were equally represented. All of the schizophrenic subjects had been so judged by an independent psychiatrist and by two of the psychiatrists (J. R. S. and R. J. D.) involved in this work, on the basis of their current clinical status and previous history. Although all had at one time shown active symptoms of schizophrenia, at the time of the investigation seven of the twenty manifested only withdrawn apathetic behaviour, with emotional blunting. Of the remaining schizophrenic subjects, one had regular cycles of catatonic symptoms and all of the others were hallucinated. No phenothiazine drug had been administered to any of the forty patients for at least 1 yr, and in some cases 18 months, but six of the schizophrenic subjects received haloperidol during a screening survey. After the screening study, eight of the patients were investigated more fully. Four were schizophrenic—three of them with active symptoms—and four were control psychiatric patients. Three healthy nurses, two female and one male, were also subjects of this latter study. Before the more detailed study haloperidol had been withdrawn for 2 weeks and was not given during the 4 week period of investigation.

The details of the analytical procedures will be reported elsewhere. The solvent extraction method was a modification of that of Friedhoff and Van Winkle[1], care being taken to avoid the use of heat and exposure to oxidation at all stages. The ion exchange procedure was essentially that used by Takesada *et al.*[2].

The solvent extraction technique yielded a pink spot precursor in the screening survey of forty patients. This was detected in the urine of four female and three male schizophrenics, and one female and three male control

patients. In addition there were some isolated examples of other pink spot substances.

The data in Tables 1 and 2 summarize the findings in the more detailed study. It can be seen that detection of the pink spot material extracted by the solvent procedure was related to fluid intake and consequent urinary output, and that the two pink spot precursors removed from the urine by the ion exchange resin were detected irrespective of fluid restriction.

The substance obtained by solvent extraction was not DMPE (3,4-dimethoxyphenethylamine) for it could be separated from the latter in the chromatographic systems and it did not fluoresce as DMPE does, using Bell and Somerville's method[3]. In a similar fashion it was shown not to be *p*-tyramine, for the two substances were separable chromatographically and the material did not give the fluorescence reaction for *p*-tyramine[4]. Because tryptamine gave an orange or red-brown colour with the reagents and could be separated chromatographically from our pink spot substance it was concluded that one could not enhance the colour of the other, as suggested by Kuehl *et al.*[5]. The only compound found to behave in a similar manner to our pink spot precursor was monoacetyl cadaverine.

The two compounds obtained by ion exchange were found to be consistently detectable, provided that the dried eluate from the ion exchange column was dissolved in methanol and chromatographed directly. If, however, the dried eluate was treated according to the method of Takesada *et al.*[2]—that is, dissolved in 1 ml. of *p*H 9·0 buffer, extracted with 5 ml. of chloroform, reduced in volume and chromatographed—then pink spots were not observed on the chromatograms in every case. One of the substances formed the same fluorophor with α-nitroso-β-naphthol as does *p*-tyramine and was isographic with *p*-tyramine in all chromatographic systems. The other was isographic with that considered to be *p*-tyramine in four of the five solvent systems, but the two were separated in the solvent system of Vogel *et al.*[6], that is, secondary butanol–formic acid 100 per cent–water. (We used paper chromatography rather than thin-layer chromatography as used by Vogel *et al.*) This second compound has not been identified. Although two other pink spot producers were detected in the urine of three subjects by ion exchange, neither of these was DMPE or the substance encountered using the solvent extraction. Similarly, solvent extraction yielded several unidentified pink spot producers from a few urine samples.

The investigations demonstrated that pink spot precursors obtained by two different extraction procedures are not necessarily identical. This was exemplified by the detection of *p*-tyramine by ion exchange in all subjects

Table 1. RELATIONSHIP OF PINK SPOT MATERIAL TO FLUID INTAKE

Subject	Sex	Age	Clinical diagnosis	Pink spot substance detected by solvent extraction: Fluid intake 1 l./24 h	Pink spot substance detected by solvent extraction: Fluid intake 2 l./24 h	Pink spot substances detected by ion exchange: Fluid intake 1 l./24 h	Pink spot substances detected by ion exchange: Fluid intake 2 l./24 h
A	F	59	Schizophrenia	+	+	+	+
B	F	37	Schizophrenia	+	+	+	+
C	M	59	Schizophrenia	−	+	+	+
D	M	47	Schizophrenia	−	+	+	+
E	M	56	Korsakow's psychosis	+	+	+	+
F	M	41	Epilepsy	+	+	+	+
G	M	32	Frontal lobe injury	−	+	+	+
H	M	67	GPI	−	+	+	+
P	M	< 30	Healthy volunteer	+	Not tested	+	+
Q	F	< 30	Healthy volunteer	+	Not tested	+	+
R	F	< 30	Healthy volunteer	+	Not tested	+	+

Table 2. CHROMATOGRAPHIC CHARACTERISTICS OF PINK SPOT MATERIAL
R_F VALUES IN VARIOUS SOLVENT SYSTEMS

Solvent system	Pink spot by solvent extraction	Monoacetyl cadaverine	First pink spot by ion exchange	Second pink spot by ion exchange	*p*-Tyramine
BUA	0·51–0·57	0·52–0·58	0·60–0·66	0·60–0·66	0·60–0·66
1s NH_3	0·69–0·79	0·69–0·79	0·79	0·79	0·79
Me Pyr	0·90–0·97	0·91–0·97	0·93	0·93	0·94
Chl–Meth	Not tested	Not tested	0·53	0·53	0·53
Bu–Form	Not tested	0·33	0·49	0·35	0·49
HVE	All migrated similar distances.				

BUA = *n*-butanol–acetic acid–water 4 : 1 : 1; 1s NH_3 = isopropanol–ammonia–water 8 : 1 : 1; Me Pyr = methanol–pyridine–conc. HCl–water 80 : 10 : 2·5 : 17·5; Chl–Meth = chloroform–methanol–acetic acid–water 65 : 30 : 2 : 3; Bu–Form = secondary butanol–formic acid 100 per cent–water 40 : 1 : 6; HVE = high voltage electrophoresis in 0·2 M phosphate buffer *p*H 6·2.

and the failure to isolate it by solvent extraction, chiefly because of the very poor partition between chloroform and urine. Boulton *et al.*[7] observed pink spots on urinary chromatograms obtained from a schizophrenic patient and four patients with Parkinsonism by the solvent extraction technique of Friedhoff and Van Winkle[1]. and proceeded to isolate a pink spot material from the urine of those patients using ion exchange. The identity of this substance was established beyond doubt as *p*-tyramine, but the inference was that the pink spot substance obtained by solvent extraction was also *p*-tyramine. We feel we have demonstrated fairly convincingly that this could not have been so.

Although the pink spot reactor we have obtained by solvent extraction behaved like monoacetyl cadaverine in several solvent systems, the two are not necessarily identical. This material, whatever its nature, is likely to be the same as that observed by Bell and Somerville[8] and by Bourdillon *et al.*[9]. This conclusion is based on the similar behaviour of our pink spot compound and that of

Bell and Somerville during high voltage electrophoresis, and the fact that "pink spot positive" urine was supplied to Bell and Somerville by Bourdillon's group. It may also have been the same as that obtained by Friedhoff and Van Winkle. As already suggested by Boulton *et al.*, several investigators probably isolated *p*-tyramine from the urine of their subjects. Takesada *et al.*[2], Kuehl *et al.*[10], Von Studnitz and Nyman[11] and Faurbye and Pind[12] all used strong cation exchangers. The failure of those workers who used the method of Takesada *et al.*[2,10,11] to observe pink spots on all of their chromatograms was probably due to two factors—the variation between individuals in quantities of *p*-tyramine excreted and the extraction into chloroform of only about 10 per cent of the *p*-tyramine present in the *p*H 9·0 buffer. The failure to detect the pink spot material observed by Von Studnitz and Nyman when subjects adhered to a glucose diet has already been explained by Vogel *et al.*[13]: a glucose diet is a diet without protein, and DeQuattro and Sjoerdsma[14] showed that excretion of *p*-tyramine is decreased during ingestion of a low-protein diet.

Perry *et al.*[15] used 4 N acetic acid to elute bases from a weak cation exchanger. Aliphatic bases such as monoacetyl cadaverine and monopropionyl cadaverine, which they identified as "pink spot" material, appeared in this eluate. Such substances are, however, displaced from the strong cation exchanger—used by us and other workers—by sodium acetate which is applied before the final eluting agent, ammoniacal ethanol. If the pink spot precursor detected by our solvent extraction procedure was an aliphatic base such as monoacetyl cadaverine, then our failure to detect it by ion exchange is explicable.

Thus we observed three pink spot substances; two were excreted by all schizophrenic and non-schizophrenic subjects investigated; although detection of the third was not demonstrable at first in every case, it was detected by an increase in fluid intake and consequent urinary output. Excretion of this latter substance by three nurses indicated that long term hospitalization was not a factor contributing to its presence in urine. It is unlikely to be of significance that we investigated only chronic schizophrenic subjects, because Bourdillon *et al.* found their pink spot on the chromatograms of both acute and chronic schizophrenics, and several workers, including Pind and Faurbye[16], examined the urine of chronic schizophrenics by the method of Friedhoff and Van Winkle and found pink spots with the R_F of DMPE on their chromatograms.

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